COMPOSITIONS AND METHODS OF USE FOR TYROSINE KINASE INHIBITORS TO TREAT PATHOGENIC INFECTION

FIELD OF THE INVENTION

The invention relates to compositions and methods for using tyrosine kinase inhibitors to treat pathogenic infection associated with or caused by host-cell interactions involving tyrosine kinases. In particular, the present invention relates to the use of Abl-family tyrosine kinase inhibitors to treat infection from microbial pathogens such as bacteria and viruses.

BACKGROUND OF THE INVENTION

The last several decades have witnessed an onslaught of deadly pathogens around the globe. A broad array of human pathogens exists, including various microbes such as bacteria, protozoa, viruses, algae, and fungi. The innate capacity to respond to selective pressures has driven the evolution of microbes and enabled them to adapt to complex and variable environments. It is perhaps no surprise, then, that infectious microbes have readily evolved mechanisms to evade our attempts to destroy them with synthetic or natural anti-microbial compounds.

The fact that microbes develop resistance at a rate that far exceeds development of new therapeutics arguably poses the single most serious public health threat in this century in both developing and developed nations. There is no denying that anti-microbial strategies have met with spectacular success over the last century. For example, antibacterial and antiviral drugs directed at targets within the pathogen have been used to save countless lives. But it is becoming increasingly evident that such success is not sustainable. To counter these drugs, bacteria and viral pathogens have evolved sophisticated mechanisms to inactivate these compounds. Examples include the pan-drug resistant strains of *Staphylococcus aureus, Klebsiella pneumonia*, and *Pseudomonas aerginosa*, and *Mycobacterium tuberculosis* (TB) among bacteria and human immunodeficiency virus (HIV) among viruses.

More worrisome still is the lack of effort on the part of pharmaceutical companies (big or small) to pursue development of new antimicrobials. Efforts to develop new antibiotics by the pharmaceutical industry by large-scale screens of chemical libraries that inhibit growth have largely failed, and new tetracycline and sulfanilamide analogs will likely engender resistance and will quickly be rendered useless. The resistance problem is compounded further by indiscriminate and inappropriate use of antibiotics and antiviral compounds without compliance measures or public health policies to reduce disease burden. With the astounding costs of clinical trials (e.g., approximately $400M to bring new tetracyclines to the market for an expected revenue of $100M), the failure to control generic sales, and the capacity to generate substantial revenues from medications for chronic illnesses there is little if any financial incentive for big pharmaceutical companies to even develop new antibiotics, and small biotechnology companies simply do not have the resources.

Even with the current level of effort there is cause for concern. Of the new drugs under development, most, if not all, will likely engender resistance quickly upon release (e.g., folate biosynthesis inhibitor Icalprim). The search for novel antiviral compounds has been somewhat more successful and largely motivated by the HIV pandemic, but drugs have been developed principally against viral targets, and mutation rates among viruses still outpaces new development. One positive development has been vaccines, which are promising for some bacterial and viral illnesses. But vaccines are not successful in all cases (e.g., in young children), and adequate resources have not been made available.

There is therefore an urgent need to develop compounds and methods effective for the prevention and treatment of pathogenic infection.

SUMMARY OF THE INVENTION

Compositions and methods for treating pathogenic infection are provided. Compositions of the invention comprise compounds that inhibit tyrosine kinases involved in pathogen-host cell interactions that are associated with or cause pathogenic infection. In some embodiments, the invention relates to the use of inhibitors of Ableson (Abl) family tyrosine kinase inhibitors such as imatinib mesylate, pyrido[2,3-d]pyrimidines, or pharmaceutically acceptable salts, enantiomers, analogs, esters, amides, prodrugs, metabolites, or derivatives thereof.

The methods of the invention comprise administering the compositions described above in therapeutically effective amounts to a patient in need thereof for treating infection by a broad array of pathogens, including microbial pathogens such as bacteria, protozoa, viruses, algae, and fungi. In particular, the invention relates to the use of these compositions to treat disease associated with bacterial and viral pathogens including pathogenic *Escherichia coli* (enteropathogenic *Escherichia coli* (EPEC), enterohemmorhagic *Escherichia coli* (EHEC), uropathogenic *Escherichia coli* (UPEC), and enteroinvasive *Escherichia coli* (EIEC)), *Helicobacter pylori*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Mycobacterium tuberculosis* (mTB), Pox viruses (including Vaccinia and variola viruses), polyoma viruses (including JC and BK viruses), Herpes viruses, cytomegalovirus (CMV), and human immunodeficiency viruses (for example, HIV-1). The compositions may be administered by any means of administration as long as a therapeutically effective amount for the treatment of pathogenic infection is delivered.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that Abl- and Src-family tyrosine kinases localize in VV actin tails. (A) Quantitation of the percentage of tails in 3T3 cells containing Abl, Arg, both Abl and Arg, or neither Abl nor Arg. (B) Quantitation of distribution of Src-family kinases in VV actin tails.

Figure 2 shows a quantitation of the distribution of ABL and Src-family kinases in VV actin tails for: (A) $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells; and (B) $Abl^{-/-}/Arg^{-/-}$ cells.

Figure 3 shows a quantitation of the effect of treatment of 3T3 cells with Abl and Src-family kinase inhibitor PD166326 prior to exposure to VV for 8 hrs. Results reflect tails in 100 infected cells. Infection was assessed by EVP staining.

Figure 4 shows that STI-571 reduces VV load in mice. Six week-old C57/B6 mice were left uninfected (no virus), or infected with 10^4 PFU/ml VV. One day prior to infection, continuous release osmotic pumps containing PBS (carrier) or STI-571 (100mg/kg/day) were surgically implanted subcutaneously. The line in each data set represents the median viral load. The data are significant ($P< 10^{-6}$) by Fisher's exact test.

Figure 5 shows quantitation of the effects of STI-X on viral replication. The percentage of infected cells is plotted, assessed by either EVP staining or the presence

of GFP-labeled virions that contained extranuclear replication centers, as measured by extranuclear DAPI staining.

Figure 6 shows that the formation and maintenance of EPEC pedestals is blocked by PD166326 and related kinase inhibitors. Graphs show the area occupied by the highest intensity pixels for EPEC treated according to the pretreatment or reversal regimens with DMSO, 10 μM PD166326, or 10 μM PP2. EPEC were cultured with either 0.1% DMSO (X) or 25 μM PD (Δ) and the OD 600 measured at the times indicated.

Figure 7 shows that PD blocks tyrosine phosphorylation of EPEC Tir but not Tir localization. Cells were treated with DMSO or PD and were left uninfected (0 h) or infected with EPEC for the times indicated. For the reversal condition, cells were left uninfected (lane 1) or infected with EPEC for 6 h, treated with PD for the times indicated, and analyzed.

Figure 8(A) shows the standard plasma curve for PD is linear from 1000 to 30 ng/ml. Figure 8(B) shows a chromatogram of mouse plasma. The mass spectroscopy readout is plotted as function of the retention time on the column. The first peak is an internal calibration standard and the second is PD.

Figure 9 shows the intercellular survival of *M. tuberculosis* after no exposure vs. exposure to STI-571 at various time points. WT stands for cells not exposed to any drug.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of compounds that inhibit tyrosine kinases involved in pathogen-host cell interactions that are associated with or cause pathogenic infection. In particular, the present invention relates to the use of tyrosine kinase inhibitors to treat or prevent diseases associated with infection from microbial pathogens, including bacterial and viral pathogens such as *Escherichia coli*, *Helicobacter pylori*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Mycobacterium tuberculosis* (TB), Pox viruses including Vaccinia and variola viruses, polyoma viruses including JC and BK viruses, Herpes viruses, cytomegalovirus (CMV), and human immunodeficiency viruses (for example, HIV-1). Particularly, tyrosine kinase inhibitors for use in the present invention include Abl-family tyrosine kinase inhibitors such as imatinib mesylate, pyrido[2,3-d]pyrimidines, or pharmaceutically acceptable salts, enantiomers, analogs, esters, amides, prodrugs, metabolites, or derivatives thereof.

The tyrosine kinase inhibitors described therein can be used in the methods of the invention to treat or prevent any pathogenic infection that is associated with or caused by tyrosine kinase-mediated host-pathogen interactions, particularly microbial infection, and more particularly viral and bacterial infection. Without being bound by theory, it is believed that the tyrosine kinase inhibitors described herein target host cells and interfere with cellular mechanisms that allow for the interaction of these host cells with pathogens and in so doing prevent the pathogenic effects caused by the pathogen. Because cellular mechanisms regulating pathogen-host interactions are remarkably conserved, it is believed that the tyrosine kinase inhibitors described herein can be applied to combat infection by a wide range of pathogens. Such pathogens include various microbes such as bacteria, protozoa, viruses, algae, and fungi. In a preferred embodiment of the present invention, the pathogens are bacteria and viruses. Advantageously, the therapeutic approach described herein targets the host, rather than the pathogen as is seen with antibiotics, and therefore decreases the likelihood of the development of pathogen drug resistance.

In one embodiment, the present invention relates to the use of tyrosine kinase inhibitors to treat or prevent bacterial infections. Such infections include those caused by members of the following genera and species: *Agrobacterium tumefaciens*, *Aquaspirillum*, *Bacillus*, *Bacteroides*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella*, *Burkholderia*, *Campylobacter*, *Chlamydia*, *Clostridium*, *Corynebacterium*

diptheriae, *Coxiella burnetii*, *Deinococcus radiodurans*, *Enterococcus*, *Escherichia*, *Francisella tularemsis*, *Geobacillus*, *Haemophilus influenzae*, *Helicobacter pylori*, *Lactobacillus*, *Listeria monocytogenes*, *Mycobacterium*, *Mycoplasma*, *Neisseria meningitidis*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Streptomyces coelicolor*, *Vibro*, and *Yersinia*. In a preferred embodiment, such infections include those caused by *Escherichia coli*, *Helicobacter pylori*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, and *Mycobacterium tuberculosis* (TB). In an other embodiment, such infections include those caused by pathogenic and/or diarrheagenic *Escherichia coli* strains, including enteropathogenic *Escherichia coli* (EPEC), enterohemmorhagic *Escherichia coli* (EHEC), uropathogenic *Escherichia coli* (UPEC), and enteroinvasive *Escherichia coli* (EIEC).

In another embodiment, the present invention relates to the use of tyrosine kinase inhibitors to treat viral infections. Such infections include those caused by members of the following virus families: *Adenoviridae*, *Arenaviridae*, *Astroviridae*, *Bacteriophages*, *Baculoviridae*, *Bunyaviridae*, *Calciviridae*; *Coronaviridae*, *Deltavirus*, *Filoviridae*, *Flaviviridae*, *Geminiviridae*, *Hepadnaviridae*, *Herpesviridae*, *Nodaviridae*, *Orthomyxoviridae*, *Papovaviridae*, *Paramyxoviridae*, *Parvoviridae*, *Phycodnaviridae*, *Picornaviridae*, *Poxviridae*, *Reoviridae*, *Retroviridae*, *Rhabdoviridae*, *Tobamoviridae*, and *Toqaviridae*. In a preferred embodiment, such infections include those caused by Pox viruses including Vaccinia and variola viruses, polyoma viruses including JC and BK viruses), Herpes viruses, cytomegalovirus (CMV), and human immunodeficiency viruses (for example, HIV-1).

In accordance with the methods of the present invention, the tyrosine kinase inhibitors described herein may be administered in combination with one another, for example, administering STI-X and STI-571, or with other compounds, particularly antipathogenic compounds. Such antipathogenic compounds include conventional antimicrobials. In other embodiments, one or more of the tyrosine kinase inhibitors described herein can be used in combination with other compounds such as cidofovir, for example, in cases related to smallpox, wherein the combination of these agents would provide for lower dosages of cidofovir to be administered, thereby decreasing the toxicity effects of this nucleoside analogue antiviral compound. Where the tyrosine kinase inhibitors of the present invention are administered as part of a

combination therapy to treat or prevent pathogenic infection, they may be administered concurrently or sequentially, in either order, with the additional compound(s).

In one embodiment, tyrosine kinase inhibitors are administered to make vaccines more effective. For example, it is well known that immunization of neonates with live viruses does not contribute to acquired immunity because maternal antibodies neutralize the vaccine (Bot and Bona (2002) *Microbes Infect.* 4: 511). In one embodiment, administration of a tyrosine kinase inhibitor of the present invention allows for safe administration of higher doses of virus to overcome antibody response and permit acquisition of cellular immunity. In another embodiment, tyrosine kinase inhibitors of the present invention facilitate immune clearance of the pathogen. For some chronic viruses (e.g., HIV and polyoma), high viral loads have been found to compromise T cell function (Welsh (2001) *J. Exp. Med.* 193:F19). Thus, lowering the viral burden could permit recovery of T cell function and thereby facilitate clearance. In another embodiment, tyrosine kinase inhibitors of the present invention permit immunocompromised individuals to be vaccinated.

The tyrosine kinase inhibitors of the present invention are for administration in a living subject or patient, including a human being or an animal such as a laboratory monkey or mouse.

Tyrosine Kinase Inhibitor Compounds for Use in the Methods of the Invention

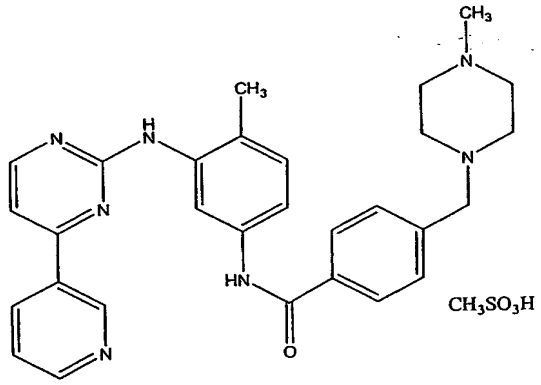
Currently, a number of families of tyrosine kinases are recognized, including Abl, Fes/Fer, Syk/Zap70, Jak, Tec, Fak, Ack, Src, and Csk. Of particular interest to the methods of the present invention are inhibitory compounds that target activity of several tyrosine kinase families, including, but not limited to, members of the Abl and Src families of tyrosine kinases. Thus, in one embodiment of the present invention, the tyrosine kinase inhibitor used to treat pathogenic infection inhibits at least members of the Abl family of tyrosine kinases, including c-Abl and c-Arg, though it is recognized that the inhibitors may also inhibit other family members.

Although ATP binding sites of tyrosine kinases are highly conserved, use of structural information obtained from X-ray crystallography and computer-assisted modeling based on kinase domain homology has led to the development of selective inhibitors. For Abl and BCR-Abl, STI-571 (also called imatinib mesylate or Gleevec®; Novartis Pharmaceuticals Corporation, East Hanover, NJ; see also U.S.

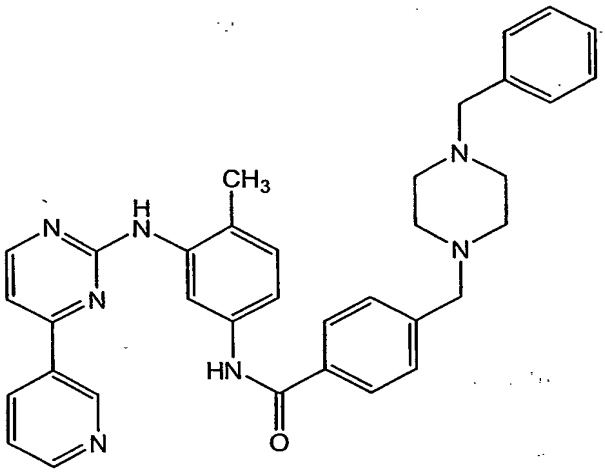
Patent No. 5,521,184, herein incorporated by reference in its entirety) is one such inhibitor with reasonably high specificity that has proven clinically useful in treating Chronic Myelogenous Leukemia (CML; Druker *et al.* (2002) *Hematology 2000* (Am. Soc. Hematol. Educ. Program):711-135; Goldman *et al.* (2001) *Blood* 98:2039). STI-571 has been used to treat stromal tumors, which are caused by dysregulation of c-Kit, a kinase with a structurally similar ATP binding site to Abl (Heinrich *et al.* (2000) *Blood* 96:925). Gleevec® is currently marketed as film-coated tablets containing imatinib mesylate equivalent to 100 mg or 400 mg of imatinib free base.

In one embodiment of the present invention, a method for preventing or treating a bacterial infection or a viral infection is provided, comprising administering a therapeutically effective amount of imatinib mesylate (STI-571) to a subject in need thereof.

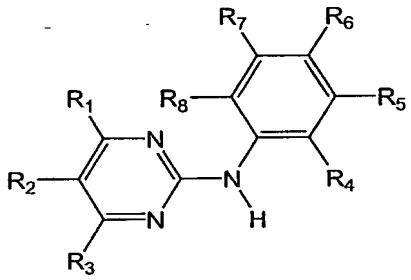
STI-571 is designated chemically as 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate, with the following structural formula:



In another embodiment of the present invention, a method for preventing or treating a bacterial infection or a viral infection is provided, comprising administering a therapeutically effective amount of a benzylated derivative of imatinib mesylate, designated STI-X, to a subject in need thereof. STI-X has the following structural formula:



According to another embodiment, the present invention encompasses compounds according to the formula:



wherein:

R_1 is 4-pyrazinyl, 1-methyl-1H-pyrrolyl, amino-, or amino-lower alkyl-substituted phenyl wherein the amino group in each case is free, alkylated, or acylated, 1H-indolyl or 1H-imidazolyl bonded at a five-membered ring carbon atom, or unsubstituted or lower alkyl-substituted pyridyl bonded at a ring carbon atom and unsubstituted or substituted at the nitrogen atom by oxygen;

R_2 and R_3 are each independently of the other hydrogen or lower alkyl, one or two of the radicals R_4, R_5, R_6, R_7, and R_8 are each nitro, fluoro-substituted lower alkoxy or a radical of the formula

$$-N(R_9)-C(=X)-(Y)_n-R_{10};$$

wherein:

R_9 is hydrogen or lower alkyl;

X is oxo, thio, imino, N-lower alkyl-imino, hydroximino, or O-lower alkyl-hydroximino;

Y is oxygen or the group NH,

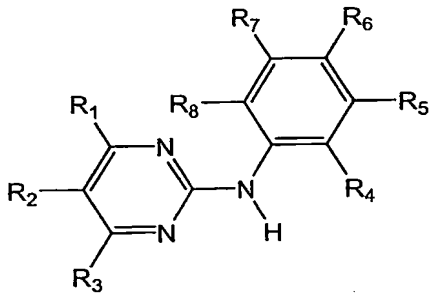
n is 0 or 1; and

R_{10} is an aliphatic radical having at least 5 carbon atoms, or an aromatic, aromatic-aliphatic, cycloaliphatic, cycloaliphatic-aliphatic, heterocyclic, or hetero-cyclicaliphatic radical;

and the remaining radicals R_4, R_5, R_6, R_7, and R_8 are each independently of the others hydrogen, lower alkyl that is unsubstituted or substituted by free or alkylated amino, piperazinyl, piperidinyl, pyrrolidinyl or by morpholinyl, or lower alkanoyl, trifluoromethyl, free, etherified, or esterified hydroxy, free, alkylated or acylated amino or free or esterified carboxy;

or a salt of such a compound having at least one salt-forming group. See, for example, U.S. Patent No. 5,521,184, herein incorporated by reference in its entirety.

According to another embodiment, the invention encompasses compounds according to the formula



wherein

R_1 is 4-pyrazinyl, 1-methyl-1H-pyrrolyl, amino-, or amino-lower alkyl-substituted phenyl wherein the amino group in each case is free, alkylated by one or two lower alkyl radicals or acylated by lower alkanoyl or by benzoyl, 1H-indolyl or 1H-imidazolyl bonded at a five-membered ring carbon atom, or unsubstituted or lower alkyl-substituted pyridyl bonded at a ring carbon atom and unsubstituted or substituted at the nitrogen atom by oxygen;

R_2 and R_3 are each independently of the other hydrogen or lower alkyl, one or two of the radicals R_4, R_5, R_6, R_7, and R_8 are each nitro, fluoro-substituted lower alkoxy or a radical of the formula

$$-N(R_9)-C(=X)-(Y)_n-R_{10};$$

wherein:

R_9 is hydrogen or lower alkyl;

X is oxo, thio, imino, N-lower alkyl-imino, hydroximino, or O-lower alkyl-hydroximino;

Y is oxygen or the group NH,

n is 0 or 1; and

R_{10} is an aliphatic hydrocarbon radical having 5-22 carbon atoms, a phenyl or naphthyl radical each of which is unsubstituted or substituted by cyano, lower alkyl, hydroxyl-lower alkyl, amino-lower alkyl, (4-methyl-piperazinyl)-lower alkyl, trifluoromethyl, hydroxy, lower alkoxy, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, lower alkanoylamino, benzolylamino, carboxy or by lower alkoxycarbonyl, or phenyl-lower alkyl wherein the phenyl radical is unsubstituted or substituted as indicated above, a cycloalkyl or cycloalkenyl radical having up to 30 carbon atoms, cycloalkyl-lower alkyl or cycloalkenyl-lower alkyl each having up to 30 carbon atoms in the cycloalkyl or cycloalkenyl moiety, a monocyclic radical having 5 or 6 ring members and 1-3 ring hetero atoms selected from nitrogen, oxygen, and sulfur, to which radical one or two benzene radicals may be fused, or lower alkyl substituted by such a monocyclic radical;

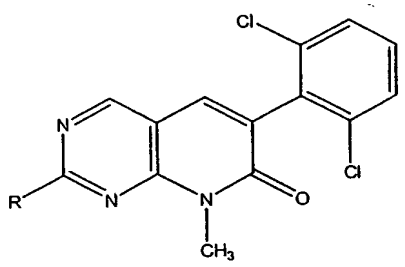
and the remaining radicals R_4, R_5, R_6, R_7, and R_8 are each independently of the others hydrogen, lower alkyl that is unsubstituted or substituted by amino, lower alkylamino, di-lower alkylamino, piperazinyl, piperidinyl, pyrrolidinyl, or by morpholinyl, or lower alkanoyl, trifluoromethyl, hydroxy, lower alkoxy, lower alkanoyloxy, halogen, amino, lower alkylamino, di-lower alkylamino, lower alkanoylamino, benzoylamino, carboxy, or lower alkoxycarbonyl,

or a salt of such a compound having at least one salt-forming group. See, for example, U.S. Patent No. 5,521,184, herein incorporated by reference in its entirety.

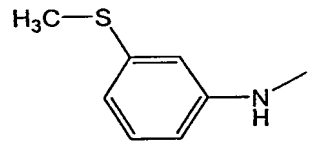
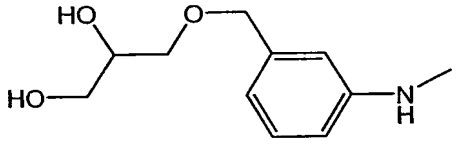
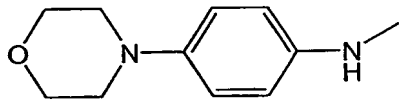
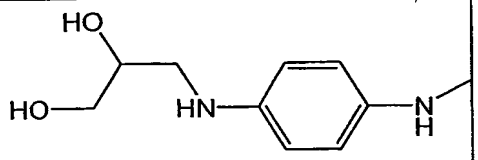
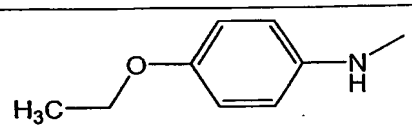
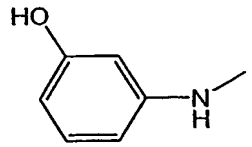
Cancer patients do develop resistance to STI-571, because inhibition of cell growth is a strong selection. This tendency to develop resistance to STI-571 has led to the search for more potent tyrosine kinase inhibitors, such as pyrido[2,3-d]pyrimidine (PD) compounds. PDs display more potency, though they differ in

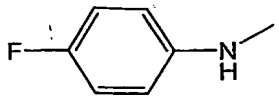
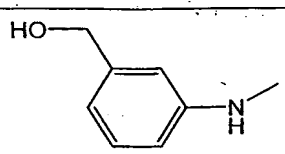
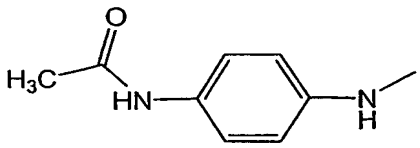
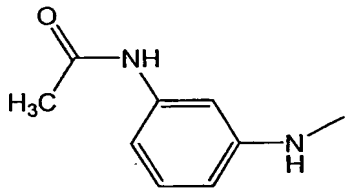
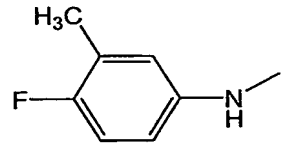
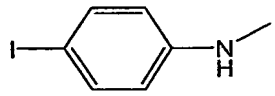
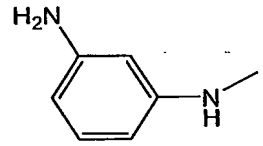
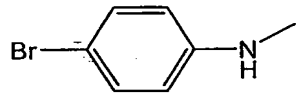
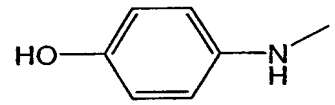
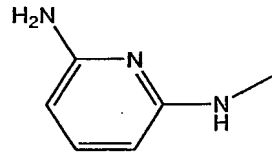
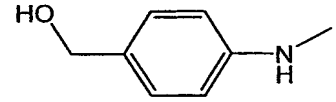
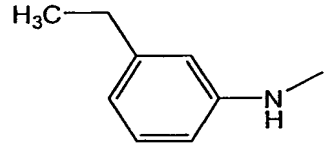
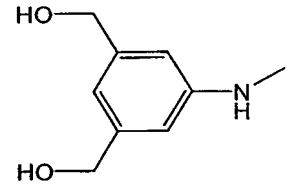
substrate specificity somewhat from STI-571 and, in addition to inhibiting Abl-family tyrosine kinases can also inhibit Src-family kinases, PDGFR, and FGFR kinases (Schindler *et al.* (2000) *Science* 289(5486):1938-1942; Wisniewski *et al.* (2002) *Cancer Res.* 62(15):4244-4255; Dorsey *et al.* (2000) *Cancer Res.* 60:3127; Kraker *et al.* (2000) *Biochem. Pharmacol.* 60:885). PDs only competitively inhibit ATP binding when the kinases are active.

In one embodiment of the present invention, a method for preventing or treating a bacterial infection or a viral infection is provided, comprising administering a therapeutically effective amount of a pyrido[2,3-d]pyrimidine to a subject in need thereof. Pyrido[2,3-d]pyrimidines that may be used according to the present invention include compounds as described in Kraker *et al.* (2000) *Biochem. Pharmacol.* 60(7):885-898; and synthesized using methods adopted from Klutchko *et al.* (1998) *J. Med. Chem.* 41:3276-3292 and Boschelli *et al.* (1998) *J. Med. Chem.* 41:4365-4377. Such compounds include those represented by the following structural formula:



wherein R equals:

PD 173955			SKI DV 2-35	
PD 173952			SKI DV 2-33	
PD 173958			SKI DV 2-89	

PD 173956			SKI DV 1-10	
PD 166326			SKI DV-M017	
SKI DV 1-10			SKI DV-M016	
PD 180970			SKI DV 2-43	
SKI DV 2-43			SKI DV 2-53	
SKI DV 2-47			SKI DV 2-71	
SKI DV 1-28			SKI DV 2-87	
SKI DV 2-45				

In another embodiment, the pyrido[2,3-d]pyrimidine selected for use according to the present invention is selected from the group consisting of:

a. PD166326 (6-(2,6-Dichlorophenyl)-2-(3-hydroxymethylphenylamino)-8-methyl-*8H*-pyrido [2,3-*d*]pyrimidin-7-one);

b. PD173952(6-(2,6-Dichlorophenyl)-8-methyl-2-(4-morpholinophenylamino)-*8H*-pyrido [2,3-*d*]pyrimidin-7-one);

c. PD173955 (6-(2,6-Dichlorophenyl)-8 -methyl-2-(3-methylsulfanyl-phenyl amino)-*8H*-pyrido [2,3-*d*]pyrimidin-7-one);

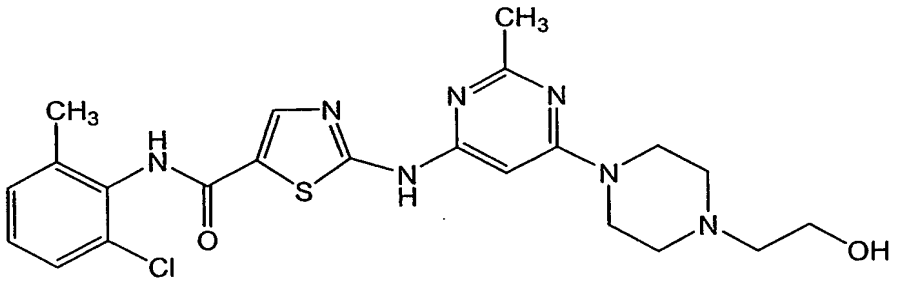
d. PD173956 (6-(2,6-Dichlorophenyl)-2-(4-fluorophenylamino)-8-methyl-*8H*-pyrido[2,3-*d*]pyrimidin-7-one);

e. PM 73958 (6-(2,6-Dichlorophenyl)-2-(4-ethoxyphenylamino)-8-methyl-*8H*-pyrido [2,3-*d*]pyrimidin-7-one); and

f. PD180970 (6-(2, 6-Dichlorophenyl)-2-(4-fluoro-3 -methylphenyl amino)- 8-methyl-*8H*-pyrido [2,3-*d*]pyrimidin-7-one).

BMS-354825 is another tyrosine kinase inhibitor that has been shown useful in cases of STI-571 resistance. BMS-354825 is a synthetic small-molecule inhibitor of SRC-family kinases that binds Abl with less stringent conformational requirements and has been shown to inhibit Abl-tyrosine family kinases with two-log increased potency relative to STI-571 (Shah *et al.* (2004) *Science* 305:399-401).

Thus, in one embodiment of the present invention, a method for preventing or treating a bacterial infection or a viral infection is provided, comprising administering a therapeutically effective amount of BMS-354825, also called [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpryimidin-4-ylamino)thiazole-5-carboxamide and having the following structure.



It is to be understood that the present invention encompasses the use not only of the specific compounds described above, but also any pharmaceutically acceptable salts, enantiomers, analogs, esters, amides, prodrugs, metabolites, or derivatives thereof.

Pharmaceutical Compositions

Because tyrosine kinase inhibitors are already the subject of drug development or are in use to treat certain cancers, data has established that they are well tolerated in humans even for extended periods (months), and are not toxic. The drugs can be ingested orally, are stable at room temperature, and are simple and inexpensive to manufacture.

In one embodiment of the present invention, a method of treating or preventing pathogenic infection, particularly microbial infection, comprises administering to a living subject in need of such treatment an effective amount of a pharmaceutical composition suitable for administration to the living subject where the pharmaceutical composition comprises: (a) at least one tyrosine kinase inhibitor in an amount effective for augmenting an inhibitable response from a host cell of the living subject responsive to at least one pathogen, particularly a microbe; and (b) a pharmaceutically acceptable carrier suitable for administration to the living subject.

In another embodiment, the present invention also relates to pharmaceutical compositions suitable for administration to a living subject, comprising: (a) at least one tyrosine kinase inhibitor in an amount effective for augmenting an inhibitable response from a host cell of the living subject responsive to at least one bacteria; and (b) a pharmaceutically acceptable carrier suitable for administration to a living subject.

In another embodiment, the present invention also relates to pharmaceutical compositions suitable for administration to a living subject, comprising: (a) at least one tyrosine kinase inhibitor in an amount effective for augmenting an inhibitable response from a host cell of the living subject responsive to at least one virus; and (b) a pharmaceutically acceptable carrier suitable for administration to a living subject.

The pharmaceutically acceptable carrier can be suitable for oral administration to the living subject, and the pharmaceutical composition is administered to the living subject orally. The pharmaceutically acceptable carrier can also be suitable for nasal administration to the living subject, and the pharmaceutical composition is administrated to the living subject nasally. Or the pharmaceutically acceptable carrier is suitable for rectal administration to the living subject, and the pharmaceutical composition is administrated to the living subject rectally. Moreover, the pharmaceutically acceptable carrier can be suitable for intravenous administration to the living subject, and the pharmaceutical composition is administrated to the living subject intravenously. Furthermore, the pharmaceutically acceptable carrier can be

suitable for inoculative administration to the living subject, and the pharmaceutical composition is administrated to the living subject inoculatively. Additionally, the pharmaceutically acceptable carrier can be suitable for hypodermic administration to the living subject, and the pharmaceutical composition is administrated to the living subject hypodermically. Thus, depending upon the pathogenic infection to be treated or prevented, the pharmaceutical composition comprising a tyrosine kinase inhibitor described herein can be administered by any suitable route, including, but not limited to, orally, nasally, buccally, sublingually, intravenously, transmucosally, rectally, topically, transdermally, subcutaneously, by inhalation, or intrathecally.

In particular, in another embodiment, these pharmaceutical compositions may be in the form of orally administrable suspensions, drinking solutions, or tablets; nasal sprays or nasal drops; or olegenous suspensions or suppositories.

When administered orally as a suspension, compositions of the present invention are prepared according to techniques well known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents known in the art. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art. Components in the formulation of a mouthwash or rinse include antimicrobials, surfactants, cosurfactants, oils, water and other additives such as sweeteners/flavoring agents known in the art.

When administered by a drinking solution, the composition comprises one or more of the tyrosine kinase inhibitor compounds described herein dissolved in drinking liquid such as water, with appropriate pH adjustment, and with carrier. The compound dissolved in the drinking liquid is an amount sufficient to give a concentration in the bloodstream on the order of 1 nM and above, preferably in an effective amount that is effective in vivo.

When administered nasally, these compositions are prepared according to techniques well known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other solubilizing or

dispersing agents known in the art (see, for example, Ansel *et al.* (1999) *Pharmaceutical Dosage Forms and Drug Delivery Systems* (7th ed.).

Preferably these compositions and formulations are prepared with suitable nontoxic pharmaceutically acceptable ingredients. These ingredients are known to those skilled in the preparation of nasal dosage forms and some of these can be found in *Remington's Pharmaceutical Sciences* (18th ed., Mack Publishing Company, Eaton, PA; 1990), a standard reference in the field. The choice of suitable carriers is highly dependent upon the exact nature of the nasal dosage form desired, e.g., solutions, suspensions, ointments, or gels. Nasal dosage forms generally contain large amounts of water in addition to the active ingredient. Minor amounts of other ingredients such as pH adjusters, emulsifiers or dispersing agents, preservatives, surfactants, jelling agents, or buffering and other stabilizing and solubilizing agents may also be present.

The formulations of this invention may be varied to include: (1) other acids and

bases to adjust the pH; (2) other tonicity-imparting agents such as sorbitol, glycerin, and dextrose; (3) other antimicrobial preservatives such as other parahydroxy benzoic acid esters, sorbate, benzoate, propionate, chlorbutanol, phenylethyl alcohol, benzalkonium chloride, and mercurials; (4) other viscosity imparting agents such as sodium carboxymethylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, polyvinyl alcohol and other gums; (5) suitable absorption enhancers; (6) stabilizing agents such as antioxidants, like bisulfate and ascorbate, metal chelating agents such as sodium edentate, and drug solubility enhancers such as polyethylene glycols.

The above nasal formulations can be administered as drops, sprays, or by any other intranasal dosage form. Optionally, the delivery system can be a unit dose delivery system. The volume of solution or suspension delivered per dose can be anywhere from 5 to 500 microliters, and preferably 5 to 200 microliters. Delivery systems for these various dosage forms can be dropper bottles, plastic squeeze units, atomizers, and the like in either unit dose or multiple dose packages. Lozenges can be prepared according to U.S. Patent No. 3,439,089, herein incorporated by reference for these purposes.

When rectally administered in the form of suppositories, these compositions may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters, or polyethylene glycols, which are solid at

ordinary temperatures, but liquify and/or dissolve in the rectal cavity to release the drug.

Dosage levels on the order of 1 mg/day or above may be useful in the treatment or prevention of pathogenic infections and related diseases within a host organism as noted herein above. In one embodiment of the present invention, a patient in need of treatment or prevention of pathogenic infection is administered a tyrosine kinase inhibitor described herein in an amount equal to or greater than about 1 mg/day, equal to or greater than about 5 mg/day, equal to or greater than about 10 mg/day, equal to or greater than about 20 mg/day, equal to or greater than about 30 mg/day, equal to or greater than about 40 mg/day, equal to or greater than about 50 mg/day, equal to or greater than about 60 mg/day, equal to or greater than about 70 mg/day, equal to or greater than about 80 mg/day, equal to or greater than about 90 mg/day, equal to or greater than about 100 mg/day, equal to or greater than about 110 mg/day, equal to or greater than about 120 mg/day, equal to or greater than about 130 mg/day, equal to or greater than about 140 mg/day, equal to or greater than about 150 mg/day, equal to or greater than about 160 mg/day, equal to or greater than about 170 mg/day, equal to or greater than about 180 mg/day, equal to or greater than about 190 mg/day, equal to or greater than about 200 mg/day, equal to or greater than about 210 mg/day, equal to or greater than about 220 mg/day, equal to or greater than about 230 mg/day, equal to or greater than about 240 mg/day, equal to or greater than about 250 mg/day, equal to or greater than about 260 mg/day, equal to or greater than about 270 mg/day, equal to or greater than about 280 mg/day, equal to or greater than about 290 mg/day, equal to or greater than about 300 mg/day, equal to or greater than about 310 mg/day, equal to or greater than about 320 mg/day, equal to or greater than about 330 mg/day, equal to or greater than about 340 mg/day, equal to or greater than about 350 mg/day, equal to or greater than about 360 mg/day, equal to or greater than about 370 mg/day, equal to or greater than about 380 mg/day, equal to or greater than about 390 mg/day, equal to or greater than about 400 mg/day, equal to or greater than about 410 mg/day, equal to or greater than about 420 mg/day, equal to or greater than about 430 mg/day, equal to or greater than about 440 mg/day, equal to or greater than about 450 mg/day, equal to or greater than about 460 mg/day, equal to or greater than about 470 mg/day, equal to or greater than about 480 mg/day, equal to or greater than about 490 mg/day, equal to or greater than about 500 mg/day, equal to or greater than about 510 mg/day, equal to or greater than about 520 mg/day, equal to or greater than about 530

mg/day, equal to or greater than about 540 mg/day, equal to or greater than about 550 mg/day, equal to or greater than about 560 mg/day, equal to or greater than about 570 mg/day, equal to or greater than about 580 mg/day, equal to or greater than about 590 mg/day, or equal to or greater than about 600 mg/day, for a patient having approximately 70 kg body weight. In some embodiments, the dose to be administered ranges from about 1 mg/day to about 1000 mg/day, including about 10 mg/day, 20 mg/day, 30 mg/day, 40 mg/day, 50 mg/day, 60 mg/day, 70 mg/day, 80 mg/day, 90 mg/day 100 mg/day, 125 mg/day, 150 mg/day, 175 mg/day, 200 mg/day, 225 mg/day, 250 mg/day, 275 mg/day, 300 mg/day, 350 mg/day, 400 mg/day, 450 mg/day, 500 mg/day, 550 mg/day, 600 mg/day, 650 mg/day, 700 mg/day, 750 mg/day, 800 mg/day, 850 mg/day, 900 mg/day, 950 mg/day, 1000 mg/day, and other such values between about 1 mg/day to about 1000 mg/day, for a patient having approximately 70 kg body weight. It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific salt or other form employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

In one preferred regimen, such dosages can be administered to a subject in need thereof by either nasal spray or by oral lozenge.

The effectiveness of using the pharmaceutical compositions of the present invention to treat or prevent a specific pathogenic infection, particularly microbial infection, may vary, for example, depending on the infectious agent, stage of infection, severity of infection, age, weight, and sex of the patient, and the like.

"Treatment" is herein defined as the application or administration of a tyrosine kinase inhibitor described herein to a subject, where the subject has a pathogenic infection as noted elsewhere herein, a symptom associated with a pathogenic infection, or a predisposition toward development of a pathogenic infection, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the pathogenic infection, any associated symptoms of the pathogenic infection, or the predisposition toward the development of the pathogenic infection. By "treatment" is also intended the application or administration of a pharmaceutical composition comprising a tyrosine kinase inhibitor described herein to a subject, where the subject has a pathogenic infection as noted elsewhere herein, a symptom associated with a

pathogenic infection, or a predisposition toward development of a pathogenic infection, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the pathogenic infection, any associated symptoms of the pathogenic infection, or the predisposition toward the development of the pathogenic infection.

The tyrosine kinase inhibitors described herein are useful in treating or preventing pathogenic infections as noted herein above. Treatment or prevention of pathogenic infection in the manner set forth herein is particularly useful for transplant patients, for example, kidney transplant patients, where emergence of pathogens, particularly polyoma viruses, for example, JC and BK, and pathogenic infection can diminish function of the transplanted organ. In like manner, HIV infection can destroy oligodendrocytes in the brain, leading to AIDS-related dementia. Thus, in addition to treating or preventing pathogenic infections as noted elsewhere herein, the tyrosine kinase inhibitors described herein can be used to control secondary infection in HIV-positive and AIDS patients and in patients receiving transplants, for example, kidney transplants, and to control AIDS-related dementia. Further, the tyrosine kinase inhibitors can be used prophylactically to prevent spread of infectious virions, for example, associated with Vaccinia infections, in immunocompromised individuals, including HIV-positive and AIDS patients and in patients receiving transplants.

EXPERIMENTAL

The following experiments examined the effects of tyrosine kinase inhibitors on the infection of host cells by pathogens, particularly bacterial and viral pathogens. Before describing these experiments in more detail, it will be helpful to provide a basic description of the pathogens studied and host-pathogen interactions.

Pathogenic *E. coli*, including enteropathogenic *E. coli* (EPEC) and enterohemmorhagic *E. coli* (EHEC), contaminate water and food supplies and cause infantile diarrhea. EPEC and EHEC are classified by NIAID as category B pathogens. In developing nations, EPEC causes sickness in some 20 million per year, killing 500,000 (Goosney *et al.* (2000) *Annu. Rev. Cell Dev. Biol.*, 16: 173). EHEC, causative agent of "raw hamburger disease," contaminates food and is associated with diarrhea and an often fatal consequence, hemolytic-uremic syndrome. EHEC possess two Shiga toxins, which cause the symptoms associated with hemolytic-uremic syndrome (Perna *et al.* (2001) *Nature*, 409(6819): 529-33).

EPEC, EHEC, and *Citrobacter (C.) rodentium* (mouse EPEC) form actin-filled membrane protrusions or "pedestals" beneath themselves on the surface of epithelial cells (Knutton *et al.* (1989) *Lancet* 2: 218; McDaniel *et al.* (1997) *Mol. Microbiol.*, 23: 399). Pedestals prevent phagocytosis, allow colonization of the host, and are required for subsequent development of disease (Goosney *et al.* (1999) *Infect. Immun.*, 67: 490; Jerse *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87: 7839). The mechanisms by which pedestals form have been extensively investigated (Kalman *et al.* (1999) *Nat. Cell Biol.*, 1: 389). The development of both pedestals and diarrhea are critically dependent on the activation of a host tyrosine kinase beneath the bacterium, which phosphorylates a bacterial protein secreted into the host cell called Tir (Kenny *et al.* (1997) *Cell*, 91: 511; Kenny (1999) *Mol. Microbiol.*, 31: 1229). Upon binding of the bacterial ligand intimin, a host signal transduction cascade is initiated that leads to pedestal formation.

The watershed event in EPEC pathogenesis is the phosphorylation of EPEC Tir (Kenny (1999) *Mol. Microbiol.*, 31: 1229). Once phosphorylated, EPEC Tir facilitates recruitment and activation of host cell proteins, including Nck, N-WASP, and Arp2/3 complex, that initiate actin polymerization to construct and brace the pedestal Kalman *et al.* (1999) *Nat. Cell Biol.*, 1: 389; Lommel *et al.* (2001) *EMBO Rep.*, 2: 850; Gruenheid *et al.* (2001) *Nat. Cell Biol.*, 3: 85619; Rohatgi *et al.* (1999) *Cell*, 97: 221).

Vaccinia virus (VV) and variola viruses are members of the *Poxviridae* family that are 95% identical in sequence (Esposito *et al.* (1990) *Poxviruses*, in *Fields Virology*, D.M. Knipe, Editor, Raven Press: New York. p. 2336; Moss (1990) *Poxviridae: The Viruses and Their Replication*, in *Fields Virology*, D.M. Knipe, Editor. Raven Press: New York. p. 2336). VV western reserve (WR) strain serves as a vaccinating agent for variola major, the cause of smallpox. VV and variola enter mammalian cells, establish extranuclear replication "factories," and produce enveloped virions (Moss (1990) *Poxviridae: The Viruses and Their Replication*, in *Fields Virology*, D.M. Knipe, Editor. Raven Press: New York. p. 2336). These virions travel to the cell surface using microtubule motors and transit into apposing cells by polymerizing actin (Ploubidou *et al.* (2000) EMBO J., 19(15): p. 3932-44; Rietdorf *et al.* (2001) *Nat. Cell Biol.*, 3(11): p. 992-1000; Ward and Moss (2001) *J. Virol.*, 75(23): p. 11651-63; Ward and Moss (2001) *J. Virol.*, 75(10): p. 4802-13; Cudmore *et al.* (1996) *J. Cell Sci.*, 109 (Pt 7): p. 1739-47; Cudmore *et al.* (1997)

Trends Microbiol., 5(4): p. 142-8). There the virions polymerize actin to propel themselves through the host cell cytoplasm and towards the plasma membrane, where they exit the cell and enter apposing cells. Formation of actin "comets" is considered critical for vaccinia to spread from cell to cell. For actin-based motility, vaccinia relies on the recruitment of host cell molecules to the surface of the particle, including tyrosine kinases. Ultimately, the host cell undergoes cytolysis thereby releasing additional infectious particles.

Tyrosine and serine/threonine kinases are important for several aspects of viral infection. Actin-based motility depends on the activity of the host cell tyrosine kinases related to c-Src and Abl, and replication at least in part depends on a viral kinase, though the precise mechanism is less well understood (Frischknecht *et al.* (1999) *Nature* 401(6756):926-929; Rempel *et al.* (1992) *J. Virol.* 66(7):4413-4426; Traktman *et al.* (1995) *J. Virol.* 69(10):6581-6587; Traktman *et al.* (1989) *J. Biol. Chem.* 264(36):21458-21461)

Upon entry of the pox virus into host cells, the virion moves to a juxtanuclear location where it replicates up to 10^4 concatameric genomes (Moss (1990) *Poxviridae: The Viruses and Their Replication*, in *Fields Virology*, D.M. Knipe, Editor. Raven Press: New York, p. 2336). The concatamers ultimately form individual enveloped particles (called intracellular mature virions (IMVs), some of which are packaged in additional membranes to form intracellular enveloped virions (IEVs; Smith *et al.* (2003) *Annu. Rev. Microbiol.*, pp. 323-342). Cytolysis releases IMVs from the cell. Prior to cytolysis, however, IEVs travel towards the host cell periphery via a kinesin/microtubule transport system (Carter *et al.* (2003) *J. Gen. Virol.*, pp. 2443-2458; Hollinshead *et al.* (2001) *J. Cell Biol.*, pp. 389-402; Rietdorf *et al.* (2001) *Nat. Cell Biol.*, pp. 992-1000; Ward and Moss (2001) *J. Virol.*, pp., 11651-11663).

To exit the cell, the IEV particle fuses with the plasma membrane of the host cell to form a cell-associated enveloped virus (CEV), leaving behind one of its two outer membranes (Smith *et al.* (2003) *Ann. Rev. Microbiol.*, pp., 323-342; Smith *et al.* (2002) *J. Gen. Virol.*, pp. 2915-2931). CEVs either detatch directly, or initiate actin polymerization to propel the particle on an actin-filled membrane protuberance towards an apposing cell and then detach (Smith *et al.* (2003) *Ann. Rev. Microbiol.*, pp., 323-342). Actin motility depends on Abl and Src family kinases whereas

detachment of CEvs to form extraceullar enveloped virus (EEV) depends on Abl family kinases (Smith *et al.* (2003) *Ann. Rev. Microbiol.*, pp., 323-342).

It is known that the protein encoded by the VV A36R gene (called A36R), located in the membrane surrounding the CEV, is required for actin polymerization and virulence (Wolffe *et al.* (1998) *Virology* pp. 20-26; Parkinson and Smith (1994) *Virology* pp. 376-390). The watershed event in actin polymerization and cell-to-cell spread is the phosphorylation of A36R tyrosine residues by a host cell tyrosine kinase (Newsome *et al.* (2004) *Science* 306:124-128; Frischknecht *et al.* (1999) *Nature* 401(6756):926-929). There is a remarkable homology between the EPEC Tir protein decribed above and the VV protein A36R, therefore using using similar but not identical host signalling factors as EPEC to polymerize actin and exit from the host cell (Frischknecht and Way (2001) *Trends Cell Biol.* 11(1):30-38).

Previous reports suggest that the mammalian tyrosine kinase c-Src localizes to virions (Frischknecht *et al.* (1999) *Nature* 401(6756):926-929). Moreover, the release of virions from microtubules and nucleation of actin to form actin tails depends on phosphorylation of A36R by Src or other kinases (Newsome *et al.* (2004) *Science* 306:124-128; Frischknecht *et al.* (1999) *Nature* 401(6756):926-929; Kalman *et al.* (1999) *Nat. Cell. Bio.* 1:389-391). Once phosphorylated, A36R facilitates detachment of kinesin and recruitment and activation of host cell proteins, including Nck, Grb2, N-WASP, and the Arp2/3 complex, which initiate actin polymerization beneath the particle (Frischknecht and Way (2001) *Trends Cell Biol.* 11(1):30-38; Moreau *et al.* (2000) *Nat. Cell Biol.*, pp. 441-448; Scaplehorn *et al.* (2002) *Curr. Biol.*, pp. 740-745). Indeed vaccinia uses mechanisms similar to those used by *Shigella flexneri* to propel itself through the host cytoplasm. For example, both *Shigella* and *Vaccinia* recruit and activate N-WASP and the Arp2/3 complex as a means of polymerizing actin (Frischknecht and Way (2001) *Trends Cell Biol.* 11(1):30-38).

Experiment 1 – Src and Abl Family Tyrosine Kinases Participate in VV Actin Motility and Release of Infectious Virions

The purpose of the present experiment was to test whether several tyrosine kinases, including members of the Src family (c-Src, c-Fyn and c-Yes) and Abl family (c-Abl and c-Arg), are required for actin motility and release of infectious EEVs. Fibroblasts lacking one or more of these kinases in conjunction with potent inhibitors

of these enzymes were used (Garcia-Echeverria *et al.* (2000) *Med. Res. Rev.*, pp. 28-57).

Methods

3T3 cells, 3T3 cells derived from $Abl^{-/-}/Arg^{-/-}$ mice, or 3T3 cells derived from $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ mice were grown on glass coverslips in DMEM containing serum and incubated for sixteen hours at 37 °C with VV (strain WR) or VVGFP-B5R at a proper amount of m.o.i. For some experiments, cells were transfected one to two days prior to infection with plasmid vectors using Fugene-6 (Roche). Abl-T315I, Argl-T314I, and Src-T338M were constructed using Quik-Change site directed mutagenesis technology. PD compounds PD166326, SKI-DRV-1-10, were synthesized as described elsewhere herein, and were indistinguishable in their effects in all assays. STI-571 was synthesized as described elsewhere herein. STI-571, PD compounds, and PP2 (Calbiochem) were dissolved in 100% DMSO. PD, PP2, or DMSO was added to cells either one hour prior to infection. For "reversal" experiments, compound or DMSO was added to cells fourteen hours after addition of VV, and the cells fixed fifteen minutes to two hours subsequently.

For immunofluorescence analysis, cells were fixed in 2% formaldehyde and permeablized in Triton-X-100. VV was recognized by staining with 4,6-diamidino-2-phenylindole (DAPI; 1 µg/ml; Sigma), and actin tails by staining with FITC-phalloidin (1 µg /ml; Molecular Probes). The primary antibodies and concentrations used in this study were as follows: α-WASP pAb (affinity purified, 1:200 dilution), α-HA mAb (3F10; .01 µg/ml, Roche), α-Nck mAb (1 µg/ml; Oncogene Research), α-Abl mAb (AB3; 0.5 µg/ml for overexpressed Abl proteins; 50 µ.g/ml for endogenous Abl proteins; 8E9; 0.05 µg/ml; Pharmingen), u-Src pAb (0.1 µg/ml; Santa Cruz), α-Arg, α-pY412, and α-TW2.3 mAb (ascites, 1:2000 for microscopy). Cells expressing exogenous c-Abl-WT were distinguished by relatively high fluorescence intensity with lower α-AbI mAb concentrations. Thus images were acquired with much shorter exposures than those used to detect endogenous c-Abl-like protein. Secondary antibodies were obtained from Jackson Immunochemicals.

For immunoprecipitation experiments, unininfected cells or cells infected with VV were washed three times with cold phosphate buffered saline and lysed for 30 minutes at 4 °C in 20 mM Tris, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitors (Complete

protease inhibitor mix; Roche). Samples were centrifuged for 20 min. at 10,000 x g. Samples were incubated with primary antibody (α-YFP, α-Src, or α-Abl) for two hours at 4 °C, and for an additional hour with protein G beads. The beads were washed with lysis buffer and analyzed by immunoblotting or used in in vitro kinase assays. For in vitro kinase assays, GST-Crk (for Abl and Arg) was used as a substrate and incubated for 30 minutes at 23 °C with 10 μM ATP in 20 μl Kinase Assay Buffer (25 mM Tris, 10 mM $MgCl_2$, 1 mM DTT) together with c-Abl, c-Abl-T315I, YFP-Arg, or YFP-Arg-T314I, previously transfected into cells and isolated on agarose beads by immunoprecipitation with α-Abl or α-YFP antibodies. Samples were then subjected to SDS-PAGE transferred to PVDF membrane and immunoblotted with α-phosphotyrosine antibody 4G10, or α-Abl mAb AB3, or α-YFP.

Images were acquired with a scientific-grade cooled charge-coupled device (Cool-Snap HQ) on a multi-wavelength wide-field three-dimensional microscopy system (Intelligent Imaging Innovations) based on a Zeiss 200M inverted microscope using a 63x N.A.1.4 lens (Zeiss). Immunofluorescent samples were imaged at room temperature using a standard Sedat filter set (Chroma) in successive 0.20 μm focal planes through the samples, and out-of-focus light was removed with a constrained iterative deconvolution algorithm.

For plaque assays, cells were seeded in 24-well dishes, grown to confluence, and incubated with VV-WR at various serial dilutions. After one hour the cells were washed to remove excess virus, and the cells were incubated for an additional 3-4 days. Cells were then fixed and stained with 20% ethanol and 4% coomasie blue to visualize plaques. For measurements of secreted EEVs, media was removed 24 hours after infection, added to uninfected 3T3 cell monolayers, and the number of plaques assessed 4 days subsequently. To determine whether different cell lines were infected (plaque reduction assays), cell monolayers were sonicated to release viral particles in the cell, and then centrifuged to remove cellular debris. The supernatant was then serially diluted and added to monolayes of uninfected 3T3 cells, and the number of plaques assessed after 3-4 days.

Six week old C57/B16 mice (Jackson laboratories) were infected by intranasal inoculation with 10^4 pfu/ml VV, a titre at which all mice died within 7 days. For mouse experiments, PD-166326 was dissolved in 30% DMSO, 30% PEG-400, and 37% saline, and STI-571 (methcylate salt) was dissolved in saline. PD-166326 (30mg/kg/day) was administered by intraperitoneal injection twice daily beginning 2-

6 hrs prior to infection and STI-571 (100mg/kg/day) was administered from subcutaneously implanted osmotic pumps. Quantitation of drug levels in the blood of control animals by HPLC/MS as described previously indicated that PD 166326 was present. The level of STI-571 in the blood was not determined. At these drug concentrations, no loss in weight or other adverse effects in uninfected animals were observed. Thus the drug appeared nontoxic.

Results

Src, Fyn, Yes, Abl and Arg Localize in VV Actin Tails. To test the hypothesis that Src and Abl-family tyrosine kinases participate in VV actin motility, it was first determined whether endogenous proteins resembling Src or Abl localized on the virion at the tip of the actin tail. 3T3 cells were exposed to VV for 15 hrs, and then stained with antibodies against Src, Fyn, Yes, Abl, and Arg. Infected cells were recognized by staining with DAPI which recognized extranuclear replication centers ("RC"), or by staining with a-TW2.3, an antibody that recognizes a vaccinia protein expressed early in infection.

The virion itself was recognized by DAPI staining or by fluorescence of a GFP-B5R fusion protein localized in the inner membrane of the virion. Actin tails may be seen as intense phalloidin staining directly apposed to the virion. An endogenous protein recognized by the antibodies against the Abl related kinase Arg was enriched at the tips of the actin tails relative to the cytoplasm. Likewise, endogenous proteins recognized by the α-Abl mAb 8E9, α-Src pAb, α-Fyn mAb, and α-Yes mAb were also enriched at the tips of the actin tails relative to the cytoplasm. Identical results were obtained with other antibodies (e.g. AB3 for Abl).

The antibodies were specific and did not recognize epitopes in cells lacking these kinases, and showed no crossreactivity with other family members as judged by transfection experiments in cells lacking Src-family or Abl-family kinases. Notably, each kinase was detectable in only a fraction of the actin tails. For example, c-Abl was detectable in some tails but not in others within the same cell. Moreover, staining with combinations of antibodies (e.g., α-Abl mAb together with α-Arg pAb), indicated that tails containing one kinase did not generally contain detectable levels of another kinase type, though both Abl and Arg kinases were evident in approximately 5% of tails.

Similar results were obtained with combinations of other anti-kinase antibodies, though because many were of similar isotype, testing of all combinations was not feasible. Figure 1A shows the percentage of tails in 3T3 cells containing Abl, Arg, both Abl and Arg, or neither Abl nor Arg. Note most tails contain one or the other kinase but few contain both. In addition, Figure 1B shows the distribution of Src-family kinases in VV actin tails. Of the five Src- and Abl-family kinases, proteins resembling c-Fyn were the most frequently observed in actin tails. Finally, no evidence was found for localization of other tyrosine kinases including PDGFR, FGFR, Lck, FAK, Ntk, Lyn, Jak1, Csk, Tyk2, and Pyk2, suggesting that localization is specific for Src- and Abl-family kinases.

To insure that the anti-kinase antibodies were indeed specific, it was next determined whether localization and distribution of exogenously expressed kinases was the same as that observed with endogenous proteins. To do this it was assessed whether yellow fluorescent protein-tagged c-Arg (YFP-c-Arg), untagged or haemagglutinin A (HA)-tagged c-Abl (HA-c-Abl) localized in actin tails. YFP-c-Arg was present in only a fraction of actin tails in transfected cells, in general agreement with results obtained with staining for the endogenous protein. Even in cells expressing high levels of YFP-c-Arg, some tails contained no YFP-c-Arg, suggesting that the localization of overexpressed kinase is specific. Additionally, co-localization was not observed for other overexpressed proteins including: Green or Yellow Fluorescent Protein, or the kinase Hck, detected with α-Hck pAb (not shown). Together these results suggest that overexpressed tyrosine kinases can specifically localize in actin tails, and that, like endogenous proteins, transfected kinases do not localize to all tails.

c-Abl or c-Arg are activated in VV actin tails. To determine whether c-Abl or c-Arg were active in VV actin tails, an antibody stain was used that recognizes the phosphorylated Y412 (α-PY412) in the activation loop domain of both proteins (Pluk *et al.*, (2002) *Cell*, 247-259). However, because the activation loop epitope recognized by the α-PY412 pAb is identical in c-Abl and c-Arg, the antibody cannot discriminate between the two proteins in a fluorescence experiment. Staining with α-PY412 was evident in the tails. Moreover, staining with α-PY412 pAb was specific for c-Abl or c-Arg, and was not evident in tails formed in cells lacking c-Abl and c-Arg.

Actin tails form on cell lines deficient in Src- or Abl-family tyrosine kinases.

To determine whether Src and/or Abl-family tyrosine kinases were necessary for actin tail formation, 3T3 cells were infected that were derived from mice lacking c-Src ($Src^{-/-}$), c-Src and Yes ($Src^{-/-}/Yes^{-/-}$), c-Fyn and c-Yes ($Fyn^{-/-}/Yes^{-/-}$) or c-Src, c-Fyn, and c-Yes ($Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$), or from mice lacking c-Abl alone ($Abl^{-/-}$), c-Arg alone ($Arg^{-/-}$), or both c-Abl and c-Arg ($Abl^{-/-}/Arg^{-/-}$). These cells were exposed to VV or GFP-VV and stained with Cy3phalloidin.

Notably, results indicated that VV retained the capacity to form actin tails in all these cell lines. No differences were apparent in the capacity to form actin tails in these cell lines compared to 3T3 cells derived from wild-type mice. In $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells, the proportion of tails occupied by Arg or Abl was somewhat higher than that occupied by these kinases in wild-type cells. Figure 2 provides a quantification the distribution of Abl- and Src-family kinases in VV actin tails in $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells, or $Abl^{-/-}/Arg^{-/-}$ cells. In $Abl^{-/-}/Arg^{-/-}$ cells, the proportion of the tails occupied by c-Src was similar to that observed in wild-type cells, though that occupied by c-Fyn was lower, and that occupied by c-Yes higher compared to wild-type cells. In spite of the differences in distribution of kinases on the tails, these results suggest that neither c-Abl, c-Arg, c-Src, c-Fyn, nor c-Yes alone appears necessary for VV actin tail formation. Moreover, these results raise the possibility that other tyrosine kinases may also localize to actin tails, and/or that localization of kinases to actin tails may be a transient or sequential process.

The observations that Abl- and Src-family kinases localize in actin tails, that Abl-family kinases are activated, but that tails formed on cell lines derived from mice lacking members of either family suggest two alternatives. First, members of either family can catalyze actin tail formation, but in the absence of any one of these kinases, another Src- or Abl-family member can suffice ("functional redundancy"). Alternatively, localization and activation of Src- and Abl-family kinases could be unrelated to actin tail formation ("localized activation"). To determine whether redundant Abl- and Src-family kinases are involved in actin tail formation, a test of sufficiency was developed based on (i) the identification of inhibitors of tyrosine kinases that block actin tail formation in wild-type cells or cells lacking particular tyrosine kinases; and (ii) the capacity of kinase mutants resistant to such inhibitors to support actin tail formation with the inhibitor present.

Inhibitors of Src and Abl-family tyrosine kinases block formation of actin tails. To distinguish functional redundancy from localized activation, the effects of tyrosine kinase inhibitors in wild-type cells was first assessed. Pyrido[2,3-d]-pyrimidine (PD) compounds competitively inhibit binding of ATP to Abl-family kinases, including c-Abl and c-Arg, and kinases with homologous ATP-binding domains including c-Src, c-Fyn and c-Yes (Dorsey *et al.* (2000) *Cancer Res.*, pp 3127-3131; Kraker *et al.* (2000) *Biochem. Pharmacol.*, pp. 885-898; Wisniewski *et al.* (2002) *Cancer Res.*, pp. 4244-4255).

3T3 cells were treated with Abl- and Src-family tyrosine kinase inhibitor PD166326 (5 μM) and then exposed to VV for 8 hrs. Cells were stained with DAPI and α-phosphotyrosine pAb to recognize infected cells, and FITC-phalloidin to recognize actin. This condition resulted in the absence of actin tails. Likewise, in cells treated with 10 μM PD and then infected with VV, no actin tails were apparent. Concentrations of PD less than 1 μM were without effect. Staining with α-TW2.3, which recognizes a VV protein expressed early in infection (Yuwen *et al.* (1993) *Virology*, pp. 732-744), was evident in cells treated with 10 μM PD, suggesting that the drug did not block viral entry. Moreover, DAPI staining or staining with an α-phosphotyrosine pAb revealed the presence of extranuclear replication centers in the presence of 10 μM PD, indicating that the drug had no detectable effect on viral replication. Quantitation of the number of infected cells with actin tails showed that treatment with 10 μM PD reduced VV tail formation at each time point by at least 50 fold compared to the carrier control (0.1% DMSO; Figure 3). Addition of 5 μM PD eight hours post infection for as little as 20 minutes also resulted in block of actin tail formation, though it is possible that PD additionally caused disassembly of extant actin tails. Compounds structurally related to PD (e.g. SKI-DV-1-10, 10 μM) were as effective as PD in blocking actin tails. The effects of PD were not due to non-specific inhibition of actin polymerization, as PD had no effect on the capacity of *Listeria monocytogenes* or *Shigella flexneri* to form actin comet tails.

PP2 and a structurally similar compound PP1 inhibit activity of Src-family kinases (Liu *et al.* (1999) *Chem. Biol.*, pp. 671-678) and have recently been recognized to additionally inhibit Abl-family kinases (Tatton *et al.* (2003) *J. Biol. Chem.*, pp. 4847-4853). Like PD, PP2 blocked actin tails at concentrations of 25 μM or greater, as reported previously (Frischknecht *et al.* (1999) *Nature*, pp. 926-929). In contrast to PD or PP2, STI-571, which inhibits Abl-family kinases but not Src-family

kinases (Schindler *et al.* (2000) *Science*, pp. 1938-1942), did not block actin tail formation in wild-type 3T3 cells, even at concentrations as high as 25 μM.

PD blocks tyrosine phosphorylation and localization of proteins required for actin tail formation. It was next tested whether PD affects localization of phosphotyrosine staining and of Nck, N-WASP, or Arp2/3 complex at the tip of the actin tail. Phosphotyrosine staining as detected with the 4G10 mAb, colocalized with virions. Likewise, staining with α-Nck mAb, α-N-WASP pAb, α-Grb2 pAb and α-Arp p41 pAb was evident around the particle at the tip of the actin tail as reported previously (not shown). When 10 μM PD was added to cells infected 15 hrs previously with GFP-VV, no localization of phosphotyrosine with the virion as detected with 4G10 mAb. Likewise, no evidence was found for localization of Nck, N-WASP, or Arp2/3 localizing with the virion. The effect of PD on phosphotyrosine was selective for that associated with motile virions because phosphorylation of targets in the replication centers recognized by α-phosphotyrosine pAb was unaffected by addition of 10 μM PD. Together, these results demonstrate that PD blocks an essential tyrosine kinase activity associated with actin tail formation but not viral replication.

Several Abl- and Src-family kinases are sufficient for VV actin motility. It was next determined which Abl- and Src-family kinases are sufficient among PD-sensitive kinases for VV actin motility.

As noted above, STI-571 had no discernable effect on VV actin motility in wild-type 3T3 cells. However, addition of 10 μM STI-571 severely limited VV actin motility in cells lacking c-Src, c-Yes, and c-Fyn, reducing the number of average number of actin tails per cell by 16 fold to ~3 per cell on average with 30% of cells having none. STI-571 had no effect on viral replication, as evidenced by extranuclear DAPI staining, or on the transit of GFP-labeled virions to the cell periphery. Moreover, the carrier for STI571, DMSO, was without effect. Together, these data suggest that: 1) kinases sensitive to STI-571, which include c-Abl and c-Arg, are sufficient to support VV actin motility; and 2) of the kinases insensitive to STI-571, c-Src, c-Fyn, or c-Yes are likely the only ones capable of supporting VV actin motility in 3T3 cells.

To determine which among Abl- or Src-family kinases was sufficient for VV actin motility, it was next assessed whether c-Abl, c-Arg, or c-Yes could support VV actin motilty in the absence of activity from other Src- or Abl-family kinases. In

particular, expression of PD-resistant alleles of c-Abl, c-Arg, or c-Yes allowed actin motility to persist in the presence of PD was tested. Previous studies have shown that mutations within the ATP binding pocket (c-Abl-T315I, c-Arg-T314I and c-Yes-T348I) disrupt Van der Waals interaction between PD and the kinases, and increases the K_i of PD from 10 nM to 1 μM as measured by in vitro kinase assay.

Next, it was tested whether these PD-resistant alleles of c-Arg or c-Abl could support VV actin tails when expressed in cells cultured in 10 μM PD. Actin tails were evident in PD-treated cells expressing YFP-c-Arg-T314I, but not in cells expressing endogenous c-Arg. Moreover, PD inhibited actin tail formation in cells overexpressing c-Arg-WT. Thus, buffering of PD, even by low affinity interactions with YFP-c-Arg-T314I, cannot account for the VV actin motility in this experiment. Not all PD-resistant tyrosine kinase alleles were capable of supporting actin tails in the presence of 10 μM PD.

In cells expressing c-Abl-T315I, actin tails were not observed in the presence of 10 μM PD. These results suggest that overexpressed tyrosine kinases, even ones that localize to the virion, do not cause nonspecific or aberrant phosphorylation of targets that support actin motility. Expression of the PD-resistant allele c-Yes-T348I, but not the wild-type allele, also supported actin tails in the presence of 10 μM PD. Together, these data indicate that c-Arg and c-Yes are sufficient among tyrosine kinases for VV actin motility, but do not rule out that other tyrosine kinases might also suffice. No evidence for the sufficiency of c-Abl in actin tail formation was found.

To determine whether other Src-family kinases were sufficient for VV actin motility, the effects of 10 μM STI-571 on cell lines lacking subsets of Src-family kinses were tested. Cells lacking c-Src and c-Yes, or cells lacking c-Fyn and c-Yes still supported VV actin motility in the presence of STI-571. Treatment with STI571 also had no detectable effects on the number of actin tails per cell. These results suggest that in addition to c-Arg and c-Yes, the Src-family kinases c-Src and c-Fyn are also sufficient for actin motility.

Redundant Src- and Abl-family kinases mediate cell-to-cell spread in vitro. To determine which tyrosine kinases participate in cell-to-cell spread, plaque assays were carried out on wild-type 3T3 cells or cells lacking various Src- and Abl-family tyrosine kinases. Infection of 3T3 cell monolayers with VV induces plaques within 4 days, though plaque morphology and size appeared somewhat more

variable and less distinct than those seen upon infection of BSC-40 cells, a commonly used cell type. Nevertheless, plaques formed in the present experiment with equal efficacy on 3T3 cells, $Abl^{-/-}$cells, $Arg^{-/-}$ cells, and $Abl^{-/-}/Arg^{-/-}$cells, $Src^{-/-}/Yes^{-/-}$cells, and $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells.

To determine whether redundant tyrosine kinases mediate plaque formation, BSC-40 cells were treated with 10 μM PD, which blocks both Abl- and Src-family kinases. In accordance with a requirement of actin tails for cell-to-cell spread, PD reduced plaque size to "pinpoints," similar to those seen in VV A36R mutants which do not readily form plaques (Parkinson and Smith (1994) *Virology*, pp. 376-390). Identical results were obtained with 10 μM PD in 3T3 cells. However, STI-571, which only blocks Abl-family kinases, did not produce significant changes in plaque size or number in 3T3 cells or BSC-40 cells, though this compound did inhibit plaque formation in $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells. Plaque reduction assays indicated that cells treated with 10 μM STI-571 or left untreated produced nearly equivalent amounts of virus after twenty-four hours, indicating that the drug had little if any detectable effect on viral replication. Together, these results provide evidence that the same redundant Abl- and Src-family kinases that mediate actin tail formation also mediate cell-to-cell spread as measured by plaque formation in vitro.

Abl-family kinases but not Src-family kinases mediate EEV release *in vitro*. To determine whether EEV formation *in vitro* was dependent on tyrosine kinases, wild-type 3T3 cells or 3T3 cells lacking various tyrosine kinases with VV were infected. Plaque assays of BSC-40 cells infected with supernatants derived from uninfected cells, VV-infected wild-type 3T3 cells, or 3T3 cells derived from animals lacking c-Src/c-Fyn/c-Yes, c-Abl, c-Arg, or c-Abl/c-Arg. Results indicated that plaques were present except when both c-Abl and c-Arg are absent, or when their activity is blocked with drug.

Supernatants were collected from cells 24 hrs after infection. At this time point, the supernatant contains plaque-forming units (PFU) composed of a significant amount of EEVs that have been released into the media (40 – 50%) and a contaminating IMVs release from lysed infected cells (Law and Smith (2001) *Virology*, pp. 132-142). The supernatant was then used to infect BSC-40 cells and plaque formation was assessed three days later.

Analysis of plaques on the BSC-40 cells indicated that supernatants from wild-type 3T3 cells, $Src^{-/-}/Fyn^{-/-}/Yes^{-/-}$ cells, $Abl^{-/-}$ cells, and $Arg^{-/-}$ cells all contained

approximately the same PFU, but supernatants from $Abl^{-/-}/Arg^{-/-}$ cells contained 5-10 fold fewer PFU. Such a decrease could not be accounted for by lower infectivity of $Abl^{-/-}/Arg^{-/-}$ cells compared to wild-type cells because the same number of plaques formed on these cells as on wild-type cells, and viral growth obtained by lysing cells 24 hours after infection and measuring plaque forming units on BSC-40 cells revealed no differences between wild-type and $Abl^{-/-}/Arg^{-/-}$ cells. Together, these results suggest that for efficient EEV release, c-Abl or c-Arg are each sufficient, and that together they are necessary.

Next, the effects of inhibitors of Abl-family tyrosine kinases on EEV formation were assessed. BSC-40 cells were infected with VV and treated with 10 μM STI571 for 24 hours. The supernatent was then collected and used to infect BSC-40 cells and plaque formation was assessed three days later. Results for BSC-40 cells were identical to results obtained with supernatant from infected 3T3 cells left untreated or treated with the drugs. Application of 10 μM STI-571 caused a ~2-fold reduction in PFU on BSC-40 cells. Treatment of 3T3 cells or BSC-40 cells with STI-571 did not by itself affect plaque formation, nor viral replication measured by plaque reduction assays. Thus, the apparent reduction in EEV number caused by STI-571 could not be attributed to block of viral entry, inhibition of cell lysis, or to inhibition of replication. Consistent with an effect of STI-571 on formation of infectious EEVs, treatment of BSC-40 cells with 10 μM STI-571 also blocked the formation of "comets" apposed to plaques, a phenomena associated with EEVs. Finally, no difference in actin tail formation or number was evident in 3T3 cells treated with STI-571. Thus, the reduction in EEV number by STI-571 was not attributable to a decrease in the number of virions reaching the cell surface. Treatment of 3T3 cells with 10 μM PD likewise reduced the number of EEVs.

Given the possibility of IMV contamination in the cell supernatent, it was next confirmed that loss of Abl and Arg activity in the $Abl^{-/-}/Arg^{-/-}$ cells or with STI-571 treatment resulted only in a reduction of infectious EEVs and not in an additional effect on IMVs. To do this, supernatants were incubated with an antibody that neutralizes IMVs, called mAb 2d5. Addition of mAb 2d5 to supernatants reduced plaque number in both 3T3 and BSC-40 cells by ~40% in agreement with previous reports (Law and Smith (2001) *Virology*, pp. 132-142). Second, addition of 10 μM STI-571 caused the same fold reduction in plaque number in the presence or absence of mAb 2D5, and the percentage reduction in plaque number with STI-571 treatment

or in $Abl^{-/-}/Arg^{-/-}$ cells was similar irrespective of the addition of mAb 2d5. Together these data suggest that Abl and Arg have little effect on IMVs, and that c-Abl and c-Arg, but not Src-family kinases, mediate release of EEVs from infected cells in vitro.

STI-571 reduces VV load in mice. To determine the role of tyrosine kinases in EEV formation and virulence in vivo, the effects of STI-571 on viral load in mice infected with VV was examined. STI-571 (100mg/kg/day dissolved in 0.9% sterile saline) or the saline carrier was delivered to mice via Azlet osmotic pumps placed subcutaneously. Twenty-four hours after insertion of the pump, some of the mice were innoculated with 10^4 pfu VV intraperitoneally. The remaining mice were left untreated. Four days post infection mice were sacrificed, and the ovaries were extracted and subjected to real time PCR to evaluate viral load. Ovaries were chosen for analysis because this organ, together with cervical tissue, have been found to contain the highest levels of virus following intraperitoneal infection (Ramirez *et al.* (2003) *Arch. Virol.*, pp. 827-839).

Viral load was measured as the number of copies of the VV UDG gene per 250 ng of DNA isolated from an ovary. In untreated animals or animals with pumps containing saline carrier, significant levels of virus were detectable in the ovaries ($\sim 10^7$ copies/250ng DNA), indicating spread of the virus to organs adjacent to the peritoneal cavity. The detection limit of the assay, determined by serial dilution, was 10 viral genomes. Treatment with 100 mg/kg/day STI-571, a concentration used in mouse leukemia models (Wolff and Ilaria, 2001), reduced viral load by 4-5 logs (Figure 4). This difference was judged statistically significant by a two-sided Fisher's exact test ($P<10^{-6}$; see Methods).

Conclusions

The results described above demonstrate that tyrosine kinases are participants in motility, release, and pathogenic infection of Vaccinia virus. In particular, Abl-family kinases, but not Src-family kinases, are required for efficient actin motility, and tyrosine kinase inhibitors that inhibit Abl-family kinases, including PD compounds, block actin motility. PD compounds and STI-571 block release of infectious virions, and STI-571 reduces viral load in VV-infected mice. In this regard, these results indicate that drugs such as PD and STI-571 are useful for the prevention or treatment of VV infection. Because Vaccinia and variola viruses are

similar, it is likely that these drugs would also have increased efficacy against variola infections in humans that cause smallpox.

Experiment 2 – STI-X Inhibits Vaccinia Replication

This experiment involved screening a small library of compounds relating to STI-571 to identify a compound affecting VV replication. A derivative of STI-571, named STI-X, was identified and examined for its effect on VV infection, replication, and motility.

Methods

Methods for cell culture and fluorescence microscopy-based plaque assays were similar to those as described in Experiment 1.

In an effort to identify compounds that affect VV replication, a library of STI-571 derivatives was constructed by making modifications to particular moieties on the molecule. These compounds were screened based upon their ability to inhibit the infection of 3T3 cells, assessed by either EVP staining or the presence of GFP-labeled virions that contained extranuclear replication centers, as measured by extranuclear DAPI staining.

3T3 cells were left untreated, or incubated with 1 μM STI-X or DMSO, the carrier. Cells were then infected with GFP-VV at an moi of 10 for 8 hours. STI-X was added at the time of infection or 6 hrs after. Eight hours after infection, cells were fixed and stained with DAPI α-Ptyr-Cy5 pAb to recognize replication centers, Cy3-phalloidin to recognize actin comet tails.

Results

The presence of GFP-virions was noted in infected cells and extranuclear replication centers absent. Ptyr staining was also absent, and no actin tails were evident. Punctate extranuclear DAPI staining that corresponded to the GFP-WR virions that initially infected the cell was evident. When STI-X was added after replication centers had formed, replication centers, and actin tails were still evident.

EVP staining and DAPI-stained extranuclear "replication factories" were evident in all untreated and DMSO-treated cells, and 90% of these cells contained actin tails, indicating that the infection was robust. GFP-labeled virions and EVP

staining were also evident in all STI-X-treated cells, indicating that STI-X had little detectable effect on viral entry. However, STI-X treatment caused a marked decrease in the percentage of cells containing DAPI-stained extranuclear viral replication factories compared to DMSO or untreated cells (100% for untreated cells compared to 4% for STI-X; Figure 2). In GFP-WR-infected cells, punctate extranuclear DAPI staining was barely visible (e.g. Fig 8A). Because the staining colocalized with GFP-WR virions, the DNA likely corresponded to virions that initially infected the cell.

STI-X-treated cells also failed to form actin tails, presumably because replication was inhibited. To test this directly, STI-X was added after replication centers had formed. Under these conditions, STI-X had no effect on replication centers (as measured by DAPI staining or α-Ptyr pAb) nor on actin motility.

Plaque assays and plaque reduction assays confirmed these microscopy observations. Plaque formation was reduced in the presence of STI-X, though the drug proved less effective when the moi was increased, a common property of antiviral drugs. The plaque assays, which were carried out over three days, indicate that the drug was well tolerated over that time period (even up to 8 days, the longest time assessed). In plaque reduction assays, the cells were infected for 24 hours in the presence or absence of STI-X. The VV was then recovered by liquid Nitrogen lysis, and the titer assessed by plaque assay in the absence of drug.

Conclusions

In summary, STI-X blocks VV replication and is useful for the prevention or treatment of VV infection.

Experiment 3 – Effect of STI-X, PD, and Combination Treatment on Aspects of VV and Variola Infection

This experiment is designed to determine the efficacy of PD, STI-X, or a combination of the two in reducing or minimizing pathogenicity in VV or Variola infected mice. C57 BL/6 mice are used for these studies. Mice are infected in a BSL2 facility to prevent infection of other mice.

STI-X and PD on VV and variola infection. Intradermal inoculation of mice with VV has been proposed to model VV vaccination in humans (Tscharke *et al.* (1999) *J. Gen. Virol.*, 80: 2751-5; Tscharke *et al.* (2002) *J. Gen. Virol.*, 83: 1977-86).

Using this model, it has been shown that intradermal inoculation on the ears of 6 week-old C57BL/6 mice with VV strain WR produces 3 mm lesions within 8 days. The lesion disappears after about three weeks indicating that the animal has developed an immune response and cleared the infection. This model was developed based on experimental groups of 5, female, age matched 6 week old C57BL/6 mice infected with 10^4 pfu intradermally on the ear, with lesion diameter measured daily over a three week time course. The present experiment follows this paradigm.

Intranasal inoculation of mice with VV has been proposed to model the normal path of variola inoculation in humans. Intranasal VV infection at an moi of 10^3 to 10^6 of 8 week old female BALB/c mice leads to dramatic weight loss, reduced activity, and ultimately death within 10 days (Reading *et al.* (2003) *J. Immunol.*, 170: 1435-1442).

The effect of PD or STI-X administered alone on lesion size (for intradermal inoculation) or mortality (for intranasal inoculation) in VV WR-infected mice is assessed. Half the mice are treated with PD or STI-X (administered via pump), and the control mice are treated equally with PBS or the drug formulation. Initially, the highest dose of PD or STI-X achievable without toxic effects is used. For mice inoculated intradermally, lesion size is measured daily. For mice infected intranasally, weight is measured daily.

At day 10 mice are sacrificed and brains and lungs are harvested. Mice losing greater than 30% of their body weight are sarcrificed immediately. Tissues are frozen and thawed tree times and sonicated, and the viral titre determined by plaque assay on 3T3 cells (Reading *et al.* (2003) *J. Immunol.*, 170: 1435-1442). Data are analyzed statistically by the nonparametric Mann-Whitney *t* test, and if PD or STI-treated mice harbor significantly different plaque forming units compared to control mice ($p < 0.01$) then it is concluded that the drug influences viral burden in infected mice. To rule out the possibility that viral invasion and proliferation is blocked by the drug formulation, or by some non-specific means, the effects of the formulation alone will be measured.

To assess the health of mice inoculated intranasally, appearance of mice are graded by a blinded observer: one point is assigned to each condition: listlessness, ruffled coat, (maximum score = 2; minimum score (robust health) = 0). In addition, body weight results are expressed as average values +/- one standard error. Treatment groups include at least five mice. Statistical analysis is calculated by the Mann-

Whitney *t* test, with $p < 0.01$ considered significant. If drug treated groups yield reduced pathology scores, it is concluded that PD therapy positively affects VV disease outcome.

Combined administration. This study assesses whether PD and STI-X administered together have the potential to provide better protection against intranasal or intradermal VV infection than either drug alone. A formulation compatible with both drugs is determined and the drug combination delivered via an Alzet pump to infect the animal intranasally or intradermally. Differences in lesion size or survivability compared to either drug administered alone or no drug are determined and analyzed as described above. Variation of the drug concentrations are required if the combination proves toxic.

Assessment of acquisition of immunity to VV. This study assesses whether STI-X or PD treatment allows effective vaccination. The drug or the carrier is administered via inoculation as described above. When the animal recovers and drug delivery has been discontinued, the animal is reinoculated. Inoculation is carried out either intradermally and the size of the ensuing scab determined, or intranasally at a dose lethal to animals not previously exposed to the virus. Scar size or mortality rates are assessed and are similar to animals not previously exposed if PD or STI-X interfere with acquisition of immunity. Alternatively, measurement of serum titres against known VV proteins and carefully dosing the drugs to avoid complications can be utilized.

Reduction of infectivity in immunocompromised patients. This study assesses whether STI-X and PD are useful in limiting VV disease in immunocompromised individuals. $Rag1^{-/-}/Rag2^{-/-}$ mice have no capacity to mount an adaptive immune response and develop severe infections. Whether intradermal inoculation with VV produces a more severe disease in these animals compared to matched wild-type animals is assessed. If so, whether administration of STI-X or PD alone or in combination serves to protect the animal from a more severe infection will be analyzed.

Experiment 4 – Entoropathogenic *E. coli* and Enteroheammorhagic *E. coli* Act Through Abl Family Tyrosine Kinases to Form Actin Pedestals

Methods

3T3 cells were grown on glass coverslips in Dulbecco Modified Eagles media (DMEM) supplemented with 10% Fetal Calf Serum and incubated for six to eight hours at 37 °C with WT EPEC (strain 2389/69) at an m.o.i. of 10, or EHEC EDL933 or EHEC-LiSTX at a m.o.i. of 40. For some experiments, cells were transfected three days prior to infection with plasmid vectors using Fugene-6 (Boehringer).

Cells were processed for immunofluorescence or Western analysis. EPEC was recognized by staining with 4'6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Sigma), and pedestals were recognized by staining with FITC-phalloidin (1 μg/ml; Molecular Probes). Before staining, some pAbs were incubated for twenty minutes with EPEC or EPEC-Δ-Tir previously fixed in formaldehyde, and then centrifuged. This procedure removed serum contaminants that nonspecifically bound EPEC.

The primary antibodies and concentrations used in this study were as follows: α-WASP pAb (affinity purified, 1:200 dilution), α-hemagglutinin A (HA) mAb (3F10; 0.01 μg/ml; Roche Diagnostics), α-Nck mAb (1 μg/ml; Oncogene Science, Cambridge, MA), α-Abl mAb (AB3; 0.5 μg/ml for overexpressed Abl proteins; 50 μg/ml for endogenous Abl proteins; 8E9; 0.05 μg/ml; BD PharMingen, San Diego, CA), α-Tir pAb (1:2000 for microscopy; 1:50,000 for Western analysis; from Jim Kaper, University of Maryland, College Park, MD), and α-Src pAb (0.1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), and α-Abl-pY412 and α-Abl-pY245 pAbs (0.1 μg/ml; Cell Signaling Technology, Beverly, MA). Cells expressing exogenous c-Abl-WT were distinguished by relatively high fluorescence intensity with lower α-Abl mAb concentrations. Thus, images were acquired with much shorter exposures than those used to detect endogenous c-Abl–like protein. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

PD compounds PD166326 and SKI-DRV-1-10 were synthesized as described elsewhere herein and were indistinguishable in their effects in all assays. PD compounds and PP2 were dissolved in 100% DMSO. For pretreatment experiments, PD, PP2, or DMSO was added to cells one hour prior to infection with bacteria. For "reversal" experiments, compounds or DMSO were added to cells five hours after addition of bacteria, and the cells fixed two hours subsequently.

Images were acquired with a scientific-grade cooled charge-coupled device on a multi-wavelength wide-field three-dimensional microscopy system (Intelligent Imaging Innovations). Immunofluorescent samples were imaged in successive 0.25 μm focal planes through the samples, and out-of-focus light was removed with a

constrained iterative deconvolution algorithm. To quantitate the effects of PD and PP2, it was noted that pedestals stained more intensely with FITC-phalloidin than actin filaments. Images were segmented on the basis of intensity. The correspondence of the highest intensity pixels to pedestals was confirmed visually. Cases where high pixel intensity and pedestals failed to coincide were adjusted accordingly. The area occupied by the highest intensity pixels was then calculated. For each condition, data were acquired from cells plated and infected identically on the same day. Each experiment was repeated five times. Some variance in maximal pedestal area was evident between experiments due to variation in plating density and infection efficiency.

Results

Transfected c-Abl and endogenous proteins reembling Abl localize in actin pedestals. Because phosphorylation of EPEC Tir triggers the pathogenic program in the host, this experiment set out to identify the tyrosine kinase involved in this process. To determine whether endogenous proteins resembling Abl localized within pedestals, 3T3 cells were exposed to EPEC or EHEC, and then stained with α-Abl monoclonal antibody (mAb) AB3 that recognizes an undefined epitope in the C-terminus. Pedestals were seen as intense actin staining, directly apposed to the bacterium. An endogenous protein recognized by α-AbI-AB3 mAb was enriched in pedestals relative to the cytoplasm. Identical results were obtained with α-Abl-8E9 mAb, which recognizes a kinase domain epitope in both EPEC and EHEC pedestals. Exogenously expressed haemagglutinin A (HA)-tagged Abl (HA-Abl), detected with the α-HA-3F10 mAb, also localized in EPEC and EHEC pedestals. The localization of Abl-like protein in pedestals was specific: neither endogenous nor exogenous Src-like protein, detected with α-Src pAb, nor green fluorescent protein fluorescence were enriched in EPEC or EHEC pedestals relative to the cytoplasm.

To determine whether endogenous proteins sharing common epitopes with Abl localized in the pedestals, 3T3 cells derived from mice lacking both Abl and the Abl-related kinase Arg were infected. Both EPEC and EHEC were still capable of forming pedestals in $Abl^{-/-}/Arg^{-/-}$ cells, and a protein recognized by α-Abl-AB3 but not by α-Abl-8E9, was evident beneath the bacterium. Together these results suggested that although Abl localizes in pedestals, other antigenically related proteins are also present.

PD compounds that block Abl and related kinases block and reverse pedestal formation. It was next determined whether Abl or Abl-like proteins were required for pedestal formation initiated by EPEC or EHEC. Because $Ab^{-/-}/Arg^{-/-}$/ cells can form pedestals. An approach was chosen that would target both Abl and structurally related proteins. Pyrido[2,3-djpyrimidine (PD) compounds competitively inhibit binding of ATP to Abl and kinases with homologous ATP-binding domains, and are being developed to treat cancers caused by dysregulated Abl (e.g. CML). With PD treatment, few attached EPEC or EHEC were evident, and little or no actin was apparent beneath bacterium that did attach, even with extended incubations (up to 8 hrs). Concentrations of PD less than 5 μM were without effect.

Quantitation of pedestals showed that PD treatment reduced EPEC and EHEC pedestal formation by 50 fold (Figure 6). Figure 6 shows area occupied by the highest intensity pixels for EPEC treated according to the pretreatment or reversal regimens with DMSO, 10 μM PD166326, or 10 μM PP2. PD analogs (SKI-DV-1-10 [DRV-1]; 10 μM) blocked EPEC pedestal formation but STI-571 (25 μM) did not. Growth of EPEC was unaffected by treatment with PD166326. EPEC were cultured with either 0.1% DMSO (X) or 25 μM PD (Δ) and the OD 600 measured at the times indicated.

Because PD compounds also inhibit some Src-family kinases, the effects of PP2, which inhibits Src-family kinases but not Abl-related kinases, was tested. PP2 at concentrations up to 100 μM, the highest tested, or the carrier DMSO (0.1%) were without effect. To test for functional redundancy between Abl and Src or other kinases, the effects of the inhibitors in $Abl^{-/-}/Arg^{-/-}$ cells was assessed. As in wild-type cells, PD but not PP2 inhibited pedestals were examined. The absence of pedestals was not attributable to a bactericidal effect of PD because no effect on growth or viability of EPEC or EHEC was apparent. The effects of PD were also not due to non-specific inhibition of actin polymerization: PD had no effect on the capacity of *Listeria monocytogenes* to attach, invade, or form actin comet tails. Together, these data indicate that Abl or-functionally redundant kinases that are sensitive to PD mediate pedestal formation.

It was next tested whether PD affected localization of Tir, Nck, N-WASP, or the Arp2/3 complex beneath attached EPEC and EHEC. Tir localized in the pedestal beneath attached bacterium, and was detectable by Western analysis after 3 or more hours of infection. With PD treatment, Tir protein remained detectable beneath attached EPEC or EHEC, despite the absence of pedestals. Although Nck, N-WASP,

and Arp2/3 complex are required for EPEC pedestal formation and, like Tir, localize in the pedestal, recruitment of Nck, N-WASP, and the Arp2/3 complex beneath EPEC was blocked by PD.

Tir phosphorylation is blocked and reversed by PD compounds. Because pedestal formation and recruitment of Nck, N-WASP, and Arp2/3 depend on Tir phosphorylated at Y474, it was next determined whether PD affected Tir phosphorylation. As shown in Figure 7, cells were treated with DMSO or PD and were left uninfected (0 h) or infected with EPEC for the times indicated. Cells were lysed and subjected to Western analysis with α-phosphotyrosine mAb 4G10, stripped, and then reprobed with α-Tir pAb. Note that Tir protein is evident after 3 h and becomes phosphorylated in DMSO-treated cells and that PD blocks Tir phosphorylation. For the reversal condition, cells were left uninfected (lane 1) or infected with EPEC for 6 h, treated with PD for the times indicated, and analyzed. Note the band corresponding to Tir becomes dephosphorylated within 5 min of adding PD.

These results suggest that PD blocks EPEC pedestal formation by blocking Tir phosphorylation, and, as a consequence, recruitment of distal signaling molecules such as Nck, N-WASP, and Arp2/3 complex that are required for actin polymerization. PD may also affect the capacity of these molecules to localize.

Abl is sufficient for Tir phosphorylation and pedestal formation in the absence of other Able-related kinases. Localization of Abl within pedestals suggested a role in Tir phosphorylation and actin polymerization, but the observation that $Abl^{-/-}/Arg^{-/-}$ cells permit pedestal formation, and the broad substrate specificity of PD suggest that other kinases might also participate. It was next determined whether Abl kinase was sufficient among PD-sensitive kinases for tyrosine phosphorylation or pedestal formation. This study took advantage of a mutation in BCR-Abl (T3151) acquired by CML patients, which renders the protein resistant to inhibition by ST1-571 or PD. The T3151 mutation was engineered into c-Abl (cAbl-3151). Expression of cAbl-T3151 in cells cultured in PD, restored EPEC and EHEC pedestal formation, as well as localization of phosphotyrosine beneath attached bacterium. Expression of c-Abl-T3151 also prevented loss of tyrosine phosphorylation in the pedestal when PD was added after pedestals had formed. Overexpression of c-Abl, even at high levels, was not sufficient to restore pedestal formation in PD, nor block loss of

phosphorylation induced by PD, suggesting that the effects of c-Abl-T3151 were due to its kinase activity rather than to low affinity binding and titration of PD.

Known substrates of Abl or Abl-related kinases localize in EPEC and EHEC pedestals. To test whether EPEC Tir is a substrate for Abl, Tir was immunoprecipitated from cells previously infected with EPEC under conditions where Tir became dephosphorylated during isolation. The presence of Tir was assessed by Western blotting with α-Tir pAb, and phosphotyrosine detected with 4G10 mAb. Addition of ATP together with purified Abl kinase to immunoprecipitated Tir resulted in tyrosine phosphorylation of Tir, and addition of PD blocked Tir phosphorylation. Whether the tyrosine phosphorylated site on Tir resembled that found in Abl targets was also assessed. CrkII is phosphorylated by Abl at Y221 and pAbs that recognize the phosphorylated Y221 on CrkII also recognize phosphorylated Tir. Thus, Abl is capable of directly phosphorylating EPEC Tir in vitro, and the phosphorylated site on Tir resembles that found in. a known Abl substrate.

Conclusions

Together, these results suggest that c-Abl activity is, among tyrosine kinases, sufficient for pedestal formation initiated by EPEC or EHEC and of EPEC Tir phosphorylation. Results with $Abl^{-/-}/Arg^{-/-}$ cells suggests that other tyrosine kinases that are sensitive to PD, and which share with c-Abl the capacity to localize in pedestals and to phosphorylate Tir or other pedestal proteins, may also suffice. Indeed, functional redundancy among tyrosine kinases is well recognized even among Abl-family members. These studies provided the first results identifying a role for tyrosine phosphorylation in EHEC pedestal formation and the first description of any tyrosine kinase sufficient for either EPEC or EHEC signalling. These results indicate that PD or related compounds may be useful to treat or prevent EPEC and EHEC infections.

Experiment 5 - *C. rodentium* Is a Useful Model of EPEC

To determine whether *C. rodentium* infection in mice is a useful model of EPEC infection in humans, the question of whether *C. rodentium* causes pedestal formation by the same mechanism as EPEC. It was found that Tir, phosphotyrosine, Nck, N-WASP, Abl, and the Arp2/3 complex all localize within C. rodentium

pedestals. Moreover *C. rodentium* failed to form pedestals on fibroblasts derived from N-WASP-deficient mice. It was next determined whether *C. rodentium* pedestals were sensitive to PD. PD in fact blocked and "reversed" *C. rodentium* pedestals. Together these results suggest that pedestals induced by EPEC and *C. rodentium* form by the same mechanism and are blocked and reversed by PD.

Experiment 6 – Administration and Detection of Drugs in Mice

To test the efficacy of the compounds of the present invention in mice, a means of introducing PD and STI into mice and detecting the compounds in serum was developed. In addition, the LD90 for VV in vivo was determined.

Intranasal inoculation with 20 µl 10^4 pfu/ml VV strain WR kills ~100% of mice within 6 days, whereas 20 µl 10^3 pfu/ml killed ~50% of the mice, in general agreement with published reports (Reading *et al.* (2003) *J. Immunol.*, 170: 1435). Intraperitoneal injection with up to 100mg/kg/day STI-571 (in saline) or up to 30mg/kg/day PD-166326 (in 31% PEG400/ 31% DMSO/ 38% Saline) was well tolerated in mice for up to 10 days, the longest time tested. For STI-571, the dose was 10 fold higher than that used to treat CML in humans, but was chosen based on the capacity of the animals to tolerate the compound, which they did (Druker *et al.* (2001) Chronic myelogenous leukemia. Hematology (Am Soc. Hematol. Educ. Program): 87; Wolff and Ilaria (2001) *Blood*, 98: 2808). Drug levels can easily be titrated to determine the minimum amount required. With drug alone, mice showed no indication of weight loss over the 10 day period, and had no overt pathology upon necropsy. Using HPLC/Mass spectroscopy it has been possible to detect PD in the serum of injected animals at concentrations as low as 30 ng/ml. The standard curve for PD is linear from 1000 to 30 ng/ml (Figure 8A). The sample volume required is 30 µl. PD was detected based on molecular weight (ion current).

The plasma sample was subjected to solid phase extraction to concentrate PD and remove plasma proteins, eluted on a Zorbax Stable Bond C8 column, and monitored in the MS (APCI positive SIM at 427). The MS readout is plotted as function of the retention time on the column. The first peak is an internal calibration standard and the second is PD (Figure 8B).

To quantitate viral load in infected mice, a real-time PCR assay was utilized to detect as few as 7 copies of VV in tissue samples. Ovaries or brains and other organs were digested with proteinase K, and the DNA extracted and purified (Qiagen). The

DNA content was normalized, and equivalent amounts of DNA were subjected to real time PCR (I-cycler) with VV WR-specific primers and a Tagman/FAM dye/quencher system. The amount of DNA in the sample was calibrated with known VV DNA standards. Using this method, the viral load in mice treated with STI-571 was 6 orders of magnitude lower than that seen in untreated mice.

Experiment 7 – *C. elegans* Screens Define Novel Drug Targets in the Host

Studies on EPEC and EHEC pathogenesis are limited by an extremely complex genome, comprising 1387 gains and 528 losses compared to *E. coli* K12, and by a lack of functional assays for many of the proposed virulence factors (Perna *et al.* (1998) *Infect. Immun.* 66: 3810). Here, a means was identified by which EPEC and EHEC pathogenesis may be studied in the nematode *C. elegans*: under specific growth conditions, the bacteria killed the worms. The killing is relevant to human disease because bacterial mutants that are nonpathogenic in humans also do not kill worms.

In a screen of mutant worms known to confer resistance to killing with other microbes, it was found that the daf-2 gene, which prolongs the lifespan of *C. elegans*, conferred resistance to killing by EPEC and EHEC (Dorman *et al.* (1995) *Genetics*, 141: 1399; Murphy *et al.* (2003) *Nature*, 424: 277). This is the first demonstrated genetic system available for studying EPEC or EHEC pathogenesis. Because both organisms can be genetically manipulated, this system offers the capacity to identify and characterize mutants in both host and pathogen. This system will allow studies on EPEC and EHEC pathogenesis in *C. elegans* that may yield identification of novel bacterial virulence factors and targets of such factors in nematode and mammalian hosts.

Experiment 8 – PD Blocks Polyoma Virus Replication In Vitro

The polyoma virus protein Middle T (MT) is essential for the virus to mount a high-level productive infection, to transform cells *in vitro*, and to generate tumors in susceptible strains of mice. MT is a type II integral membrane protein that recruits, binds, and activates the host cell kinases c-Src, c-Fyn, and c-Yes. A number of *in vitro* and *in vivo* studies have established that the capacity of MT to bind and activate these tyrosine kinases is required for the viral growth promoting and oncogenic

functions. Virtually all humans are persistently infected with each of the two known human polyomaviruses: JCV and BKV.

Although human polyoma viruses do not encode an MT protein, a homologous protein small T (ST) does exist. Other viruses, such as EBV, also target Src kinase (Longnecker *et al.* (1991) *J. Virol.* 65: 3681).

In this experiment, the effect of PD and STI-571 on the cytopathic effects of Polyoma virus on 3T3 cells was assessed. Both PD and STI-571 inhibited cytopathic effects. Monolayers of 3T3 cells were left uninfected or infected for 5 days with Polyoma virus. Cells in the infected group were divided into conditions: DMSO (the carrier for PD); 10 μM STI-571; and 1 μM PD166326. Polyoma infection caused cell death in the DMSO group, but addition of STI-571 and PD reduced the extent of killing. These results demonstrate that these compounds are therefore useful as inhibitors of Polyoma virus infection.

Experiment 9 - STI-571 Blocks HIV Replication In Vitro

The present experiment examined the effect of STI-571 on HIV replication. Culture macrophages were infected with either media, HIV-Bal, various dosages of STI-571, or various dosages of STI-571 combined with HIV-1 Bal. Viral replication (measured by p24 levels) was reduced by up to 4 fold in a dose dependent fashion by addition of STI-571 (Table 1). These results demonstrate that STI-571 is useful as an inhibitor of HIV infection.

Table 1. Effects of STI-571 on p24 production 7 and 14 days after infection of macrophages with HIV-1 Bal.

Culture Condition	P24(pg/ml) day 7	P24(pg/ml) day 14
Cells + media	< 10	< 10
Cells + HIV-1 Bal	322 +/- 28	956 +/- 34
Cells + STI-571 1 μM	< 10	< 10
Cells + STI-571 5 μM	< 10	< 10
Cells + STI-57I 10 μM	< 10	< 10
Cells + HIV-1 Bal + STI-571 1 μM	287 +/- 31	744 +/- 27
Cells + HIV-1 Bal + STI-571 5 μM	212 +/- 44	556 +/- 28
Cells + HIV-1 Bal + STI-571 10 μM	127 +/- 22	245 +/- 31

Experiment 10 - Development of Tyrosine Kinase Inhibitors

The present experiment was designed to develop new potent inhibitors for a number of biologically relevant tyrosine kinases (Abl, PDGFR, and Src). STI-571

and pyrido[2,3-d]pyrimidines were derivitized (Goosney *et al.* (2000) *Ann. Rev. Cell Dev. Biol.*, 16: 173; Knutton *et al.* (1989) *Lancet* 2: 218). These derivatives were screened on the basis of different desired characteristics, including optimization of solubility, mere pharmacokinetic and pharmacodynamic properties, as well as specificity in blocking kinases affecting microbial pathogenesis but not those affecting immune clearance. Based upon such screening, STI-571 was identified (see Experiment 2 above). These results demonstrate that derivatizing STI-571 and pyrido[2,3-d]pyrimidines can yield molecules with novel specificities or desirable in vivo properties.

Experiment 11 - Effects of Tyrosine Kinase Inhibitors on Pathogenesis of TB In Vitro

The present experiment addressed whether selected tyrosine kinase inhibitors can affect pathogenesis of *Mycobacterium tuberculosis* (TB), the etiologic agent of tuberculosis. Invasion of TB into a cultured human macrophages (line THP-1) was carried out essentially as described in Miller and Shinnick (2001), *BMC Microbiol.*, 1: 26. Briefly, TB cultures was be added to the cells for between 30 minutes and two hours. Actinomycin D was then be added to the cultures to kill any bacteria remaining extracellularly. The actinomycin D was then washed away, and the cells lysed to release invaginated bacteria. The lysate was then be plated on bacterial plates, and the number of recovered colonies counted. The experiments were performed with or without addition of PD, STI-571 at concentrations ranging from 100 nM to 10 μM, concentrations that have proven effective in other EPEC and VV assays.

Colony counts were an indication of whether invasion was inhibited. Cell growth assays and trypan blue exclusion were used to verify that the macrophages were not adversely affected by the drugs. Results indicated that STI-571 increases the intercellular survival of *M. tuberculosis* (Figure 9). These results indicate that tyrosine kinase inhibitors are effective in inhibiting TB infection.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific

terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation. Further, it must be noted that as used in this specification and the appended embodiments, the singular forms "a," an" and "the" include plural referents unless the context clearly dictates otherwise.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.